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CANCER RESEARCH

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Morphological and Biological Characteristics of X-Ray Induced Transplantable Ovarian Tumors*

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The observation that total body irradiation in female mice is frequently if not inevitably followed by the development of ovarian tumor (8) has been confirmed and extended by several investigators (13, 15, 17, 19). The induction period of the tumors is very long. While regenerative changes begin after 4 to 6 months (5), tumor-like growth does not appear before about 7 months; large tumors are infrequent and metastases are rare.

Six years ago experiments were begun to ascertain by transplantation studies the neoplastic character of the hyperplastic nodules that occur following irradiation and to secure pure lines of neoplastic cells of different sorts. Transplantations are suitable to study the morphogenic potencies of the ovarian tumor cells of different types, their interrelationship and the secondary changes they produce. Thus, 16 ovarian tumor strains have been studied, of which a luteoma has already been described *in extenso* (9). The successful transplantation of granulosa tumors and their striking secondary changes have been recorded in preliminary reports (6).

It is the purpose of this paper to survey the characteristics of the 16 strains of transplantable ovarian tumors and to find out what they contribute toward the understanding of ovarian tumor problems. Fifteen attempts to transplant x-ray induced tumors were unsuccessful in the first passage. Most of these were done under unfavorable conditions and in a small number of mice, and will not be reviewed.

Tumors arising in irradiated ovaries are complex. Most transplanted neoplasms are of the granulosa cell type; a smaller number are luteomas and tubular adenomas. All of these types are frequently present in the same x-rayed ovary, and the type of tumor particles used for transplantation, the growth rate of the different elements, and probably other factors residing in host tissue and donor determine which and how many types of transplantable neoplasms are isolated from a single irradiated ovary. Less commonly encountered neoplasms are hemangiomas and endotheliomas, (two of which resembled chorio-epitheliomas) and sarcomas. Only two types of cells thus far transplanted were found to be associated with hormone production: granulosa cell tumors cause morphological changes indicative of the production of estrogens, and luteomas of progestins.

Increase of blood volume goes invariably with granulosa tumor (11, 20) and for the hypothetical substance responsible for this, the name plethorin will be used.

MATERIALS AND METHODS

The ovarian tumors were cut up in Tyrode's solution and implanted through various routes, including the spleen, liver, and the anterior chamber of the eye. Since the tumors were induced in F_1 (Ak/Rf) hybrids, the transfers were made routinely in young (2 to 3 months old) mice of this stock. The organs studied were fixed in Zenker-formol or formol solution, embedded in paraffin or cut in the frozen state, stained routinely by hematoxylin and eosin and occasionally with Mallory's

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anilin blue, Masson's trichrome, Sudan III, and other special procedures as will be named. The irradiation factors and induction of the tumors have been described (7).

Strain I: Granulosa Type

The animal from which this strain originated received 175 r when 40 days old, was painted with methylcholanthrene twice a week during 4 weeks (beginning one day after irradiation), and was killed 12 months after irradiation. The right ovary was replaced by a white-yellow-brown tumor, 18 × 13 mm. in the two greatest diameters. The left ovary appeared normal. A metastatic node was found in the liver.

The ovarian tumor was of the granulosa cell type. In places the cells formed amorphous masses, in places cords and follicle-like structures. The cytoplasm was scanty and the nuclei showed a coarse stippled chromatin pattern (Fig. 1). In some masses and follicles, the cells liquefied into an eosinophilic transparent substance. There were many edematous and necrotic areas.

In the first passage, the same structures were found in the tumors (Fig. 2); but the follicular formations of Graafian type were more abundant (Figs. 3 and 4).

In the second subpassage, deeply stained fibroblast-like cells appeared. In parts granulosa cells with elongated nuclei showed a bundle-like arrangement, some were detached, resembling plasma cells.

In the third subpassage antrum-like structures occurred in the solid masses (Fig. 4). They contained desquamated cells, or a transparent eosinophilic liquid, or red blood corpuscles. The tubules were lined with cuboidal cells (Fig. 5) some with papillary formations. In some tumors, the low cylindrical cells formed rows along the capillaries.

In the fourth subpassage, the structures remained the same. Areas of degeneration (Fig. 6), fibrosis and necrosis were present in almost all tumors. The histological picture did not change in the subsequent passages and the ability of the tumor cell to form follicles was retained after a year of subpassages.

Transplantation and biological behavior:—The results of all transplantations are summarized in Table 1. The per cent of takes after subcutaneous injection of normal animals fluctuated after an increase in the first and second subpassages; it dropped and rose again in the fifth subpassage. The males seemed to be slightly more susceptible (55 per cent) than the females (42 per cent). Intrasplenic injections were successful in all of 8 normal mice of both sexes.

The latent period of the tumors did not change in a constant direction. There was an increase until the fourth subpassage, followed by a decrease. The average was 30 days with a range of 7 to 85 days. The rate of growth did not seem to parallel the latent period.

The secondary changes produced by this strain as well as by other granulosa strains are of at least two kinds: a) estrogenic; b) a dilatation of sinusoidal system with blood volume rise, hereafter referred to as plethoric. These effects have been mentioned in previous papers (11, 20) and are being further investigated currently (14).

The estrogenic influence was manifest from the first subpassage on. Previous irradiation or gonadectomy of the host did not materially alter them.

All routes of introduction save the intrasplenic had these effects. The latter does not usually go with hormonal changes except when the tumor adheres to structures adjacent to the spleen. The congestive changes became manifest during the second subpassage and persisted until the final subpassage. They were independent of the site of injection and in the females a slightly greater tendency of manifesting this change was recorded.

Intraabdominal hemorrhages occurred in 12 cases, due mainly to the rupture of the exceedingly congested liver, spleen, or adrenal. The tumors exhibited malignant properties: subcutaneous tumors penetrated in the peritoneal cavity and metastasized to the liver (10 cases); lungs (15 cases); kidneys (1 case), and ovary (1 case). Only in two cases was the tumor the source of the hemorrhage. Hemorrhage into the thorax was also seen. In one case pleural hemorrhage from pulmonary metastasis was the cause of death.

In addition to these two main changes (estrogenic and plethoric) most animals had a high degree of relative anemia and splenomegaly with marked extramedullary hemopoiesis.

Strain II: Granulosa Type

The original animal was irradiated with 175 r when she was 40 days old. She was painted with methylcholanthrene for 5 weeks, twice weekly (7) and was killed 7 months after irradiation. The right ovary was replaced by a hemorrhagic yellow-grey tumor of 15 × 13 × 9 mm. The uterus was about 3 mm. thick. The tumor was composed of granulosa-sarcoma cells with coarse and fine stippled nuclei, forming masses, and follicular structures (Fig. 7). In places, the tumor cells regressed leaving a stroma rich in capillaries.

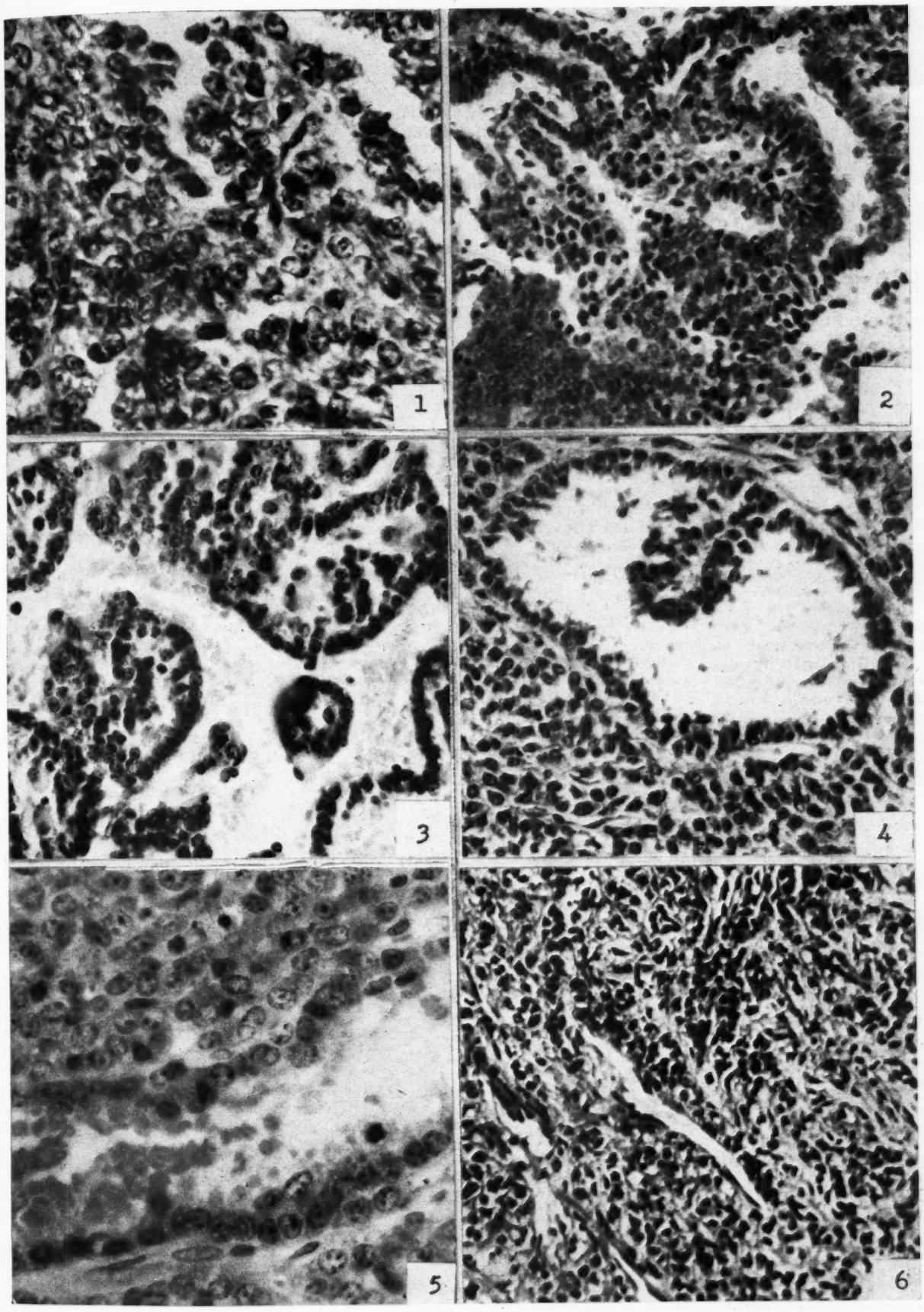
In the first passage the tumors were of pure granulosa cell type, forming solid masses and cords. Areas of fibrosis were present. In the second passage, sarcomatous forms appeared. There were abundant necrotic areas, many of which were calcified, some ossified. The microscopic picture did not change essentially in the course of subpassages, although in some tumors, cords in others masses or follicular arrangements were predominant. The sharpness of stippling of nuclei was variable; it was usually present (Figs. 8 and 9). A cavernous hemangioma developed in one of the recipients of the fourth subpassage.

Figures 10 to 12 show metastatic lesions in lung, kidney, and liver respectively. Sudanophile fat was abundant in granulosa cells in areas of degeneration and scant if any in "healthy" cells. Acid-fast granules (ceroid) appeared in granulosa cells in areas of degeneration (10).

Tubular necrosis in the kidney as illustrated in Figure 22 was a frequent finding.

Transplantation and biological behavior:—The average per cent of takes was 47 per cent in both sexes. No significant change occurred in the course of the subpassages.

The latent period of the tumors increased from an initial 17 days to 55 days in the fourth subpas-



FIGS. 1 to 6.—Strain I.

FIG. 1.—Solid masses of granulosa cells. Original tumor. Mag. × 800.

FIG. 2.—Solid masses, rows and pyknotic granulosa cells. First subpassage. × 350.

FIG. 3.—Follicle-like structures. Sixth subpassage. × 280.

FIG. 4.—An antrum follicle with a cumulus oophorus-like formation. Fourth subpassage. × 280.

FIG. 5.—Solid masses and rows of granulosa cells. Fifth subpassage. × 400.

FIG. 6.—Replacement of degenerating tumor by connective tissue. Fourth subpassage. × 220.

sage. In the fifth and sixth subpassages it dropped back to 17 days. The shortest latent period was 5 days, the longest 172 days. The growth rate of the tumors dropped in the second passage, suddenly increased in the fourth subpassage.

Estrogenic effects on the tumor-bearing hosts were recorded in 11 of 27 males and 6 of 27 females. Congestive changes were often encountered. Both estrogenic and congestive changes were manifest also in irradiated recipients. The site of the tumor had no influence on the congestive changes.

The transplanted tumors were malignant. Two penetrated into the abdominal cavity, 5 metastasized to liver, 8 to lung, 1 to kidney, and 1 to adrenal.

Abdominal hemorrhage occurred in 7 cases. This was due either to an intraabdominal tumor mass or to the rupture of the highly congested liver, spleen, or adrenal.

Strain III: Granulosa, Luteoma, Ademona, and Sarcoma Lines

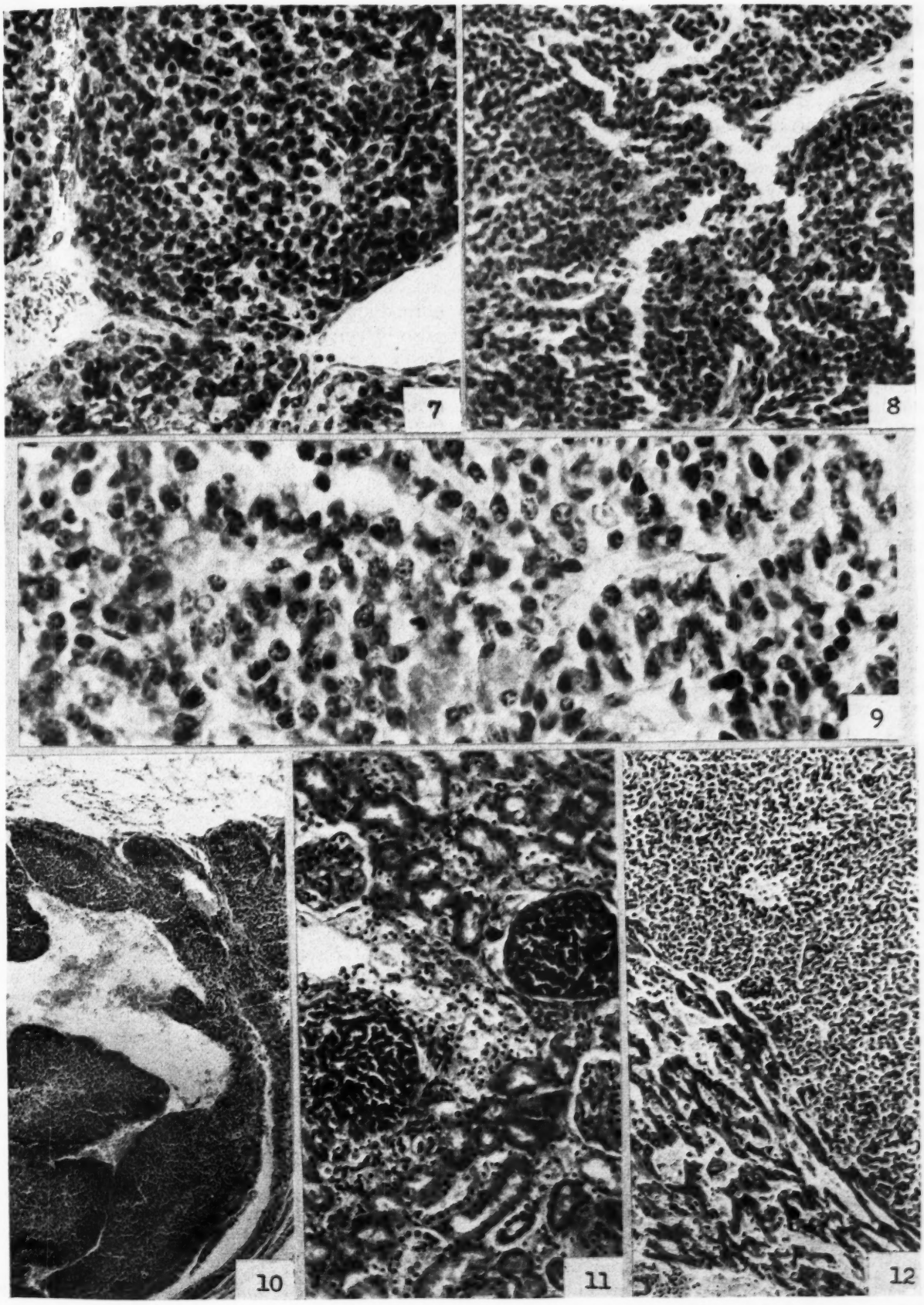
The original animal was irradiated with 175 r at 47 days of age and painted with methylcholanthrene twice during one week. Ten months later the left ovary was replaced by a yellow-

TABLE 1

TRANSPLANTATION DATA ON OVARIAN TUMORS INDUCED BY X-RAYS

Strain	Type of tumor	Number of Passages	Number of Subpassages	Sex	Normal: Subcutaneous	Normal: Intrasplenic	Gonadectomized: Subcutaneous	Gonadectomized: Intrasplenic	Gonadectomized: Intrahepatic	Pre-irradiated: Subcutaneous
I	Granulosa			F	47/112 (42%)	3/3 (100%)	6/15 (40%)	3/3 (100%)	1/3 (33%)	
		34	10	M	119/218(55%)	5/5 (100%)	8/16 (50%)	2/3 (66%)	3/4 (75%)	4/7 (57%)
II	Granulosa			F	14/30 (46%)		2/10 (20%)			25/69(36%)
		15	6	M	8/17 (47%)					26/62(42%)
III	Granulosa			F	52/108 (49%)		11/29(38%)	0/5		9/13 (69%)
		27	9	M	51/114 (45%)		9/9 (100%)	0/1		0/4
	Luteoma			F	12/13 (92%)					
		3	1	M	9/10 (90%)					
	Fibroscarcoma	1	1	F	7/9 (78%)					
	Granulosa			F	27/64 (42%)		7/12 (58%)			7/19 (37%)
		16	4	M	26/68 (38%)					0/3
IV	Adenoma			F	1/9 (11%)		2/3 (66%)			
		4	2	M	1/2		0/3			
V	Granulosa			F	99/323 (31%)	3/13 (23%)	19/78(24%)	11/18(66%)	11/14(78%)	6/19 (31%)
		80	15	M	104/267(39%)	6/12 (50%)	26/55(47%)	18/33(55%)	4/7 (57%)	0/2
	Granulosa			F	0/10		1/8 (13%)			
		4	1	M	0/7		4/19 (21%)			
VI	Adenoma-granulosa			F	0/31		11/14(78%)			3/12 (25%)
		13	4	M	2/25 (8%)		4/4 (100%)			4/7 (57%)
	Fibrosarcoma	1	1	F	1/6 (17%)					
VII	Adenoma			F	0/10		13/24(54%)	0/2		1/4 (25%)
		13	3	M	1/21 (5%)		6/14 (43%)	0/2		
VIII	Adenoma			F			2/12 (17%)			2/5 (40%)
		5	2	M	2/11 (18%)					
IX	Luteoma			F	103/205(50%)	2/2 (100%)	12/18(66%)	1/4 (25%)		6/8 (75%)
		39	10	M	75/169 (44%)	1/2 (50%)	9/23 (39%)	1/4 (25%)		2/6
X	Adenoma			F	0/13		6/10 (60%)			0/2
		5	2	M	2/4					
XI	Luteoma-granulosa	17	2	F	20/81 (25%)					
				M	24/57 (42%)					
XIII	Adenoma-granulosa	5	2	F	4/14 (29%)					
				M	7/18 (39%)					
	Fibrosarcoma	9	3	F	11/44 (25%)					
				M	9/28 (32%)					
XIV	Granulosa	15	3	F	5/47 (11%)	0/3	10/11(91%)			
				M	9/68 (13%)	1/2				
XV	Adenoma	8	2	F	1/26 (4%)	2/2				
				M	5/30 (17%)	2/2				
XVI	Adenoma	5	2	F	0/7					
				M	4/25 (16%)					
XVII	Adenoma	3	1	F	0/11					
				M	5/19 (26%)					
	Leuteoma	6	1	F	1/14 (7%)					
				M	7/26 (27%)					
XVIII	Adenoma	2	1	F	1/5 (20%)		3/5 (60%)			
				M	0/2					

The ratios indicate the number of positives over the total number of mice injected.
Subcutaneous implantation of Strain I to 20 Ak mice and intravenous and intracardiac injections to 10 homologous Rf/Ak mice were not successful.
Intraperitoneal injections made to 20 mice of Strain II were successful in only one and were not successful in 4 mice of Strain III.
With Strain I, intratesticular injections were successful in 2 of 8 mice, intramuscular in 3 of 4, and intravenous in 1 of 3.
Intraocular implantations of Strain V were successful in 21 of 30 mice of the homologous stock, in 13 of 17 mice of the heterologous Rf stock, and in 1 of 13 rats. Intraocular implantations of Strain IX were successful in 2 of 6 mice of the heterologous Rf stock. Intraocular implantations of Strain XIII were unsuccessful in 6 mice of the homologous stock.



FIGS. 7 to 12.—Strain II.

FIG. 7.—Solid follicle-like structures. The tumor is made up of nodules of granulosa cells. Original tumor. × 200.

FIG. 8.—Solid growth of similar cells without follicle formation. Fourth subpassage. × 200.

FIG. 9.—Higher power of Figure 8, from the proximity of a "cleft." Fourth subpassage. × 300.

FIG. 10.—Metastasis to lung. Sixth subpassage. × 50.

FIG. 11.—Metastasis to kidney. Fourth subpassage. × 150.

FIG. 12.—Metastasis to liver. Fourth subpassage. × 100.

grey partially hemorrhagic and necrotic tumor, measuring 2 × 5 × 1.5 cm. The right ovary was yellow and 1 × 2 mm. in size. The uterus was 3 mm. thick.

The left ovarian tumor used for transfer was composed of granulosa cells, imitating Graafian follicles. Calcium and other crystalloid deposits and foreign body giant cells were seen in the margin of necrotic areas. The tumor cells stained with Gomori's technique were acid phosphatase negative and slightly positive for alkaline phosphatase.

In the first passage granulosa cells formed masses and follicular structures; there were large areas of necrosis and edema. Hortega stains showed a network of reticulum fibers, in places coarse, in places fine-meshed. Sudanophilic fat was present in cells in the edematous areas.

The accumulation of some fluid material separating widely the tumor cells (Fig. 13) and some degenerative changes were characteristic of this strain. When connective tissue growth was massive the microscopic picture resembled scirrhous carcinoma (Fig. 14). Calcification and ossification of stroma was common in older tumors (Fig. 15). In one animal of the fifth passage a well differentiated fibrosarcoma replaced the tumor (Fig. 16). This was readily transplantable.

One tumor in the first passage showed a typical luteoma and upon subpassages a pure luteoma line was secured (Fig. 17). A third line yielded a fibrotic tubular adenoma. Thus, this strain was divided in three lines of which the granulosa and luteoma lines were carried for several generations; the adenoma line was lost.

Some secondary changes in granulosa tumor-bearing mice included: massive atrophy of testes (Fig. 18), of ovary (Fig. 19), feminization of the kidney (Fig. 21), and of the submaxillary gland (Fig. 20). Sections of kidneys frequently showed an advanced necrosis of proximal tubules.

Transplantation and biological behavior:—The granulosa line of this strain showed a fluctuation in the per cent of takes. The highest ratio of takes (around 65 per cent) was in the fourth, fifth, and sixth subpassages and the average somewhat below 50 per cent in both sexes. The pre-irradiation had no significant effect. All of the gonadectomized males but only 38 per cent of the females developed tumors (Table 1).

The luteoma line had a higher percentage of takes than the granulosa line, (about 91 per cent) and was equal in males and females.

The latent period of the granulosa line decreased during the subpassages from 81 days to 17 days, the longest was 138 days. The growth rate did not significantly change during the subpassages; the tumors reached a dimension of about 33 × 20 mm. in 2½ months.

The granulosa tumors were malignant. Twelve lung, 5 liver, 6 intraperitoneal, 1 splenic, and 1 ovarian metastases were noted. Two subcutaneous tumors penetrated into the abdominal cavity. Massive hemorrhages were common. Thus 7 cases of intraperitoneal, 1 intrathoracic, 1 intrasplenic, 1 intrahepatic, and 1 intraadrenal hemorrhages were recorded. Four mice had generalized edema, one with ascites. Anemia and splenomegaly were present in most of the granulosa tumor-bearing animals.

The luteoma line had an average latent period of 42 days, the shortest being 16, the longest 74 days.

Estrogenic changes were recorded in only 15 of 33 granulosa tumor-bearing females and in 2 of 6 gonadectomized females. Of 25 normal males, 19 were recorded as exhibiting estrogenic changes while 2 gonadectomized males did not show them.

Congestive changes were recorded in 19 of 25 normal and in 5 of 6 gonadectomized females. The ratio of congestive changes was 18/25 in normal and 2/2 in gonadectomized animals. Thus, there was no marked difference between males and females and normal and castrated recipients.

Strain IV: Granulosa, Adenoma, and Endothelioma Lines

The original animal received 300 r when 41 days old. Sixteen months later a laparotomy was performed and a piece from each ovarian tumor was transplanted separately.

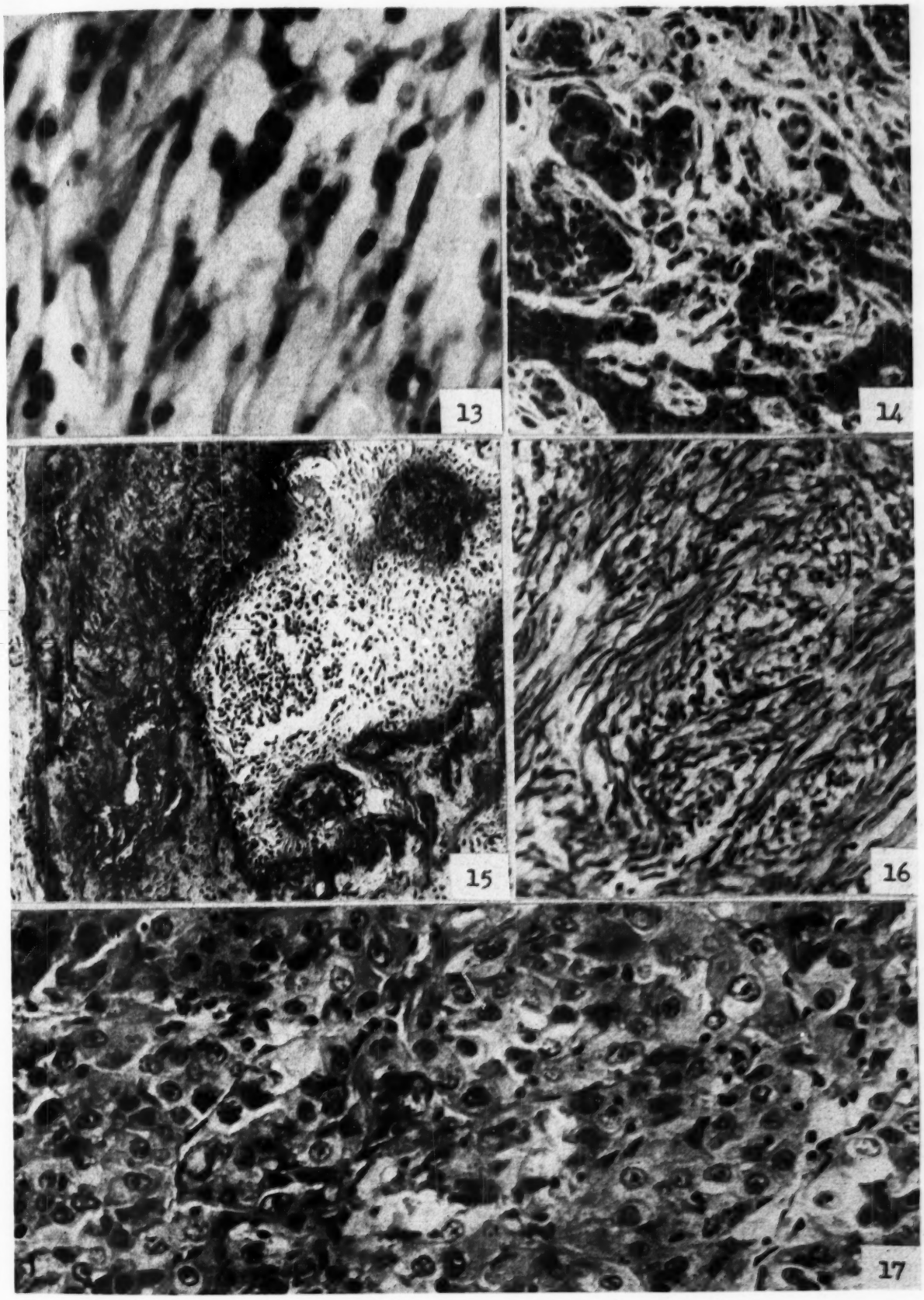
The left ovarian tumor was of the granulosa cell type with areas of massive type of growth, cords (Fig. 23), and follicle-like structures surrounded by "oat-shaped" cells. In a narrow area germinal epithelial tubules were seen with a stroma of spindle-shaped cells. The right ovary had a tubular adenoma with few granulosa cells among the tubules.

During the subpassages of this tumor, granulosa cells gained an upper hand in one line. Granulosa cells were seen inside the basement membrane of tubules as well as in spaces among them as shown in Figure 44. These differentiated into "anovular" follicles (Fig. 28) or the secondary proliferation products of germinal epithelium of Brambell and Parkes (5). Transfers from this line were unsuccessful.

The passages from the left ovarian tumor invariably gave granulosa cell tumors with areas of trabecular and follicular arrangements. Mixed with typical granulosa cells were encountered elongated cells with scanty cytoplasm and an "oat-shaped" nucleus, rich in chromatin (Fig. 26). These are frequently referred to as theca cells. They formed bundles along vessels and around follicular structures. In places they outgrew the spherical granulosa cells. The presence of the two types of cells with carcino-sarcoma-like appearance was characteristic of this line. Accumulations of fatty material with formation of lutein-like cells are shown in Figures 24 and 25. A tubular adenoma line as illustrated in Figure 27 soon vanished in the course of subpassages.

Several tumors had capillaries with cavernous dilatation. The tumor cells among them appeared to vanish. Thrombi had formed in these capillaries. In some tumors connective tissue overgrew the tumor cells among the capillaries; in others an active proliferation of endothelial cells appeared to take place. This was carried on as a pure line believed to be either a chorio-epithelioma or hemangio-endothelioma (Fig. 29). Accepting the majority opinion of specialists and because of lack of hormonal production, we are considering it as an endothelioma. It will be described separately with other endotheliomas (1).

Transplantation and biological behavior:—The granulosa line of this strain had at first a higher per cent of takes: in the first passage 57 per cent in males, 83 per cent in females; in the next passage 91 per cent in males and 100 per cent in females.



FIGS. 13 to 17.—Strain III.

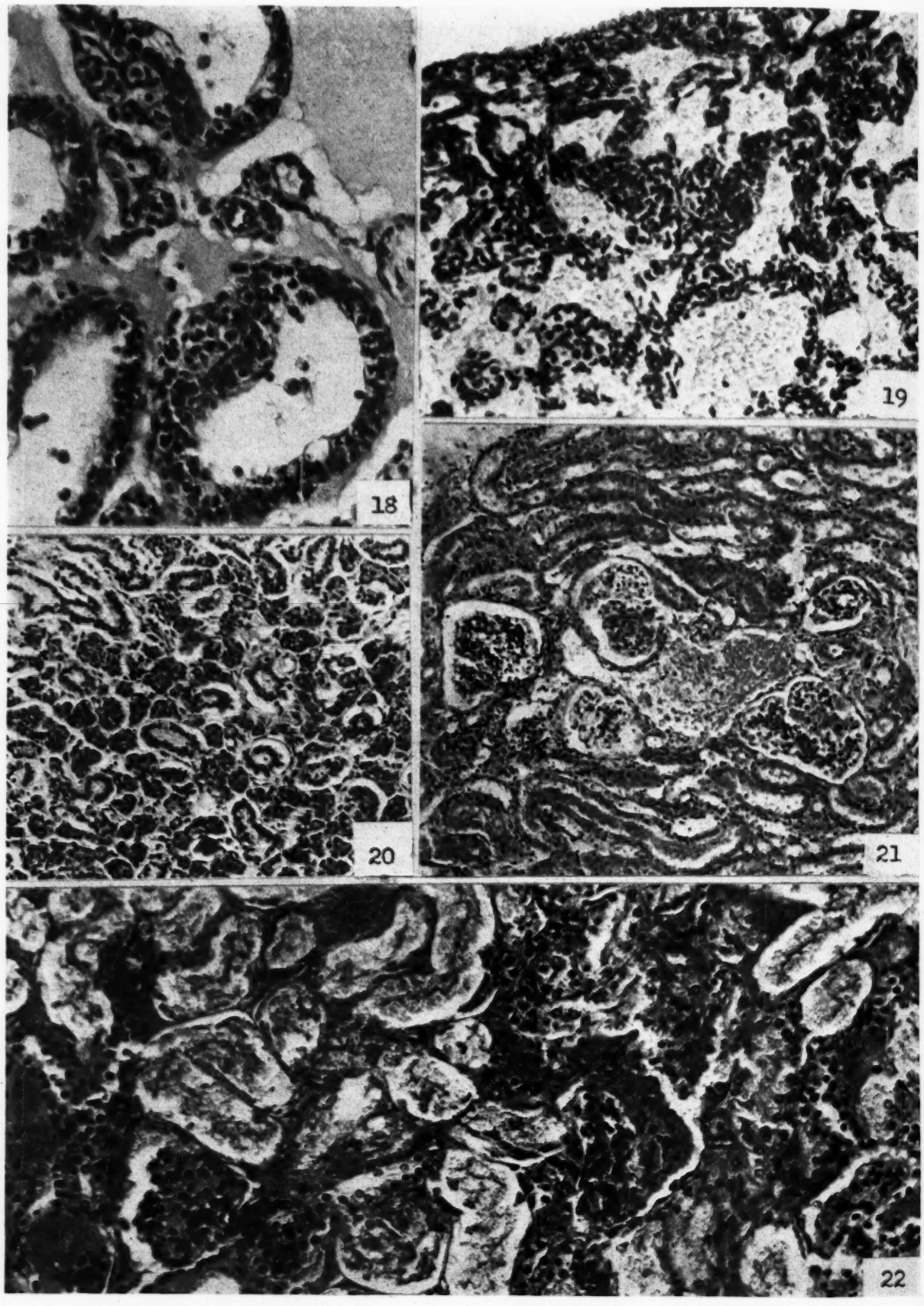
FIG. 13.—Degenerative changes with elongation and separation of cells probably by some secretion product. Sixth subpassage. × 400.

FIG. 14.—Connective tissue overgrowths; resemblance to scirrhous carcinoma. Third subpassage. × 220.

FIG. 15.—Beginning ossification of stroma. Fifth subpassage. × 80.

FIG. 16.—A well-differentiated fibrosarcoma replacing the tumor. Fifth subpassage. × 280.

FIG. 17.—Luteoma line appearing in the first subpassage. × 500.



FIGS. 18 to 22.—Strain III. Secondary changes in tumor-bearing mice.

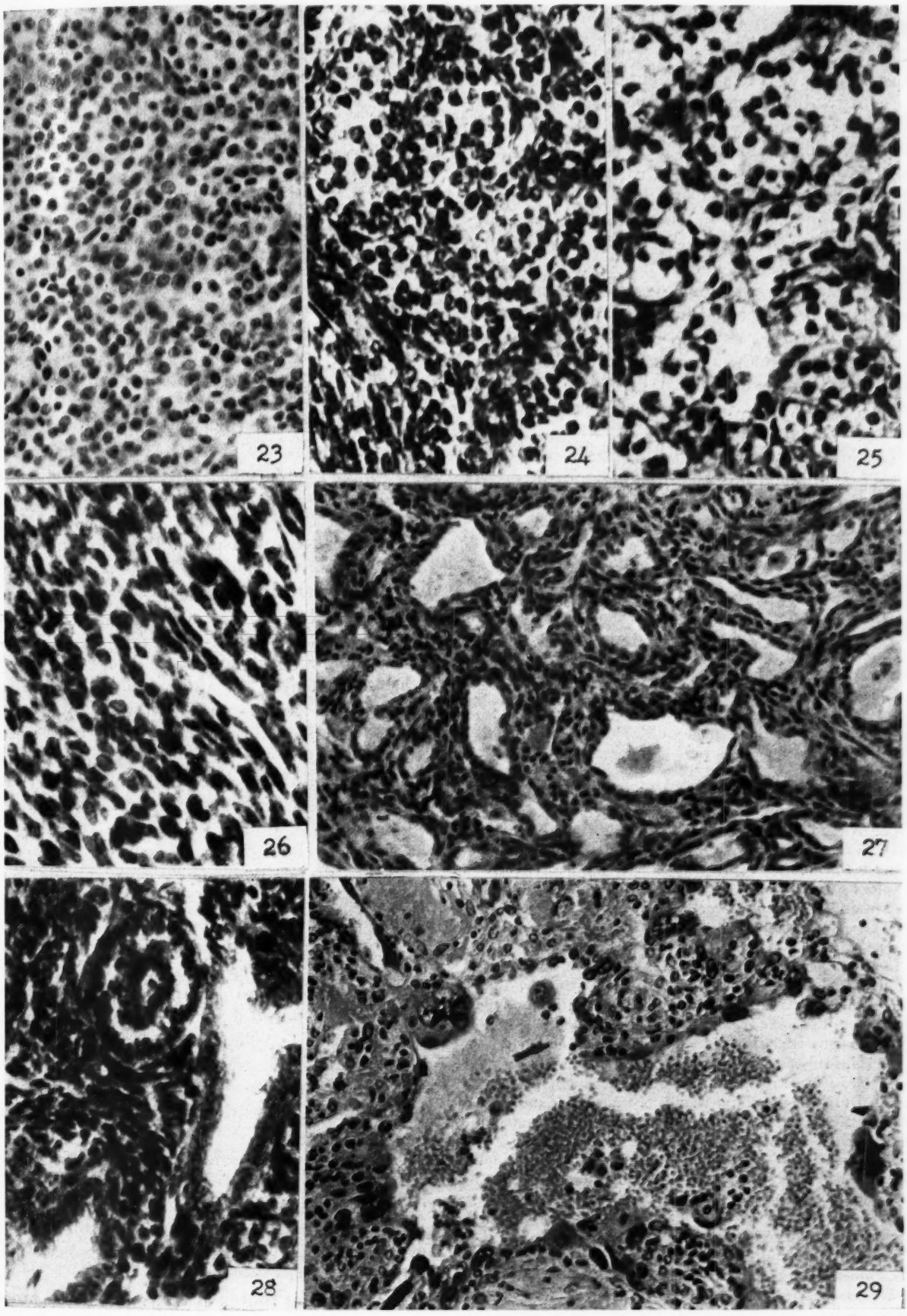
FIG. 18.—Marked atrophy of testis. Sixth subpassage. × 360.

FIG. 19.—Marked atrophy of ovary. Seventh subpassage. × 250.

FIG. 20.—Effect on submaxillary gland of a male mouse; collapsed tubules with expansion of acini. Sixth subpassage. × 80.

FIG. 21.—Feminization of parietal layer of Bowman's capsule of a male mouse. Fourth subpassage. × 150.

FIG. 22.—Necrosis of proximal tubules, male mouse. Fifth subpassage. × 400.



FIGS. 23 to 29.—Strain IV.

FIG. 23.—Solid granulosa growth. Original tumor. × 280.

FIG. 24.—"Fattened" granulosa cells; suggestive of early partial luteinization. Second subpassage. × 300.

FIG. 25.—Luteinization of granulosa cells. Fourth subpassage. × 300.

FIG. 26.—Sarcoma-like forms of granulosa cells. Third subpassage. × 400.

FIG. 27.—Tubular adenoma line. Second subpassage. × 150.

FIG. 28.—Complex anovular granulosa follicle, tubular adenoma, and spindle cells. First subpassage. × 300.

FIG. 29.—Endothelioma line with chorio-epithelioma-like areas. Third subpassage. × 150.

This dropped in the subsequent subpassage to 30 per cent for males and 22 per cent for females.

Pre-irradiation did not significantly influence the success of inoculations. The tubular adenoma grafts were successful in but a small per cent of animals.

The latent period in the granulosa line was about 66 days. The growth rate of granulosa tumors was very slow, the tumors reaching about 3 × 2 mm. in 1 month. Nevertheless, they were malignant: 2 penetrated into the abdominal cavity, 1 metastasized to lung, and 9 from spleen to liver. Several tumors regressed in the third subpassage. The growth rate of the tubular adenoma was also slow, the tumors reaching about 9 × 6 mm. in 11 months.

The hormonal activity of this strain was slight, only two gonadectomized females exhibiting estrogenic changes. No congestive changes were observed.

Strain V: Granulosa Type

The original animal was irradiated with 300 r when 41 days old. Sixteen months later a grey tumor 6 mm. in diameter replaced the right ovary. This was transplanted. The left ovary was 2 mm. in diameter and yellow. No hyperplasia of uterine horns was observed. The mammary glands were atrophic.

The original tumor showed a mixed composition, the major parts being of granulosa type, the remaining a tubular adenoma. The granulosa cells formed follicles and tubules, in some of the latter the lining cells were partly granulosa and partly germinal epithelium.

The left ovary was a tubular adenoma with ciliated epithelium, pigmented (ceroid) cells, and a small number of granulosa cells or ovariocytes (21) among the tubules. The uterine horn showed a slight cystic hyperplasia.

In the first subpassage the tumor showed a massive type of granulosa growth (Fig. 31). In many places the cells formed rows, in others follicular structures (Fig. 30), with an eosinophilic substance in some. Ossification was seen in the stroma (Fig. 34).

In the subsequent subpassages the chromatin arrangement in nuclei changed from granular chromatin structure into a more homogeneous chromatin arrangement with slight condensation in the juxta-membranous parts of the nucleus (Fig. 33). Cavernous dilatation of the capillaries was characteristic. In the third subpassage granulosa cells of a sarcomatous type were seen in some recipients. In others, the insular form of Varangot (22), namely large islands of tumor cells surrounded by connective tissue, have been observed. In many tumors the nuclei resembled those of the germinal epithelium (Fig. 31). In the sixth subpassage, microfollicular formations (Varangot) and spaces resembling Call-Exner bodies were seen. In the tenth and later subpassages "oat-shaped" cells were numerous. In some recipients of the twelfth subpassage some tumor cells were detached and resembled histiocytes and plasma cells.

In a second granulosa cell line of this strain, the granulosa cells formed trabecular structures separated with wide connective tissue septa. The cells tended to form rows as in the "moire-silk" pattern of Varangot (Fig. 32) and papillary proliferations of somewhat cylindrical cells. Extensive bone formations were seen in the stroma of some recipients (Fig. 34). These tumors were stone hard and it was surprising to note on microscopic examination "healthy" tumor cells side by side with "healthy" bone (Fig. 34).

In the sections stained with Sudan III, the cells in fibrotic areas and those with pyknotic nuclei were heavily laden with fat. The "healthy" cells did not contain fat, not even discrete droplets. In the line with "moire-silk" pattern, some recipients showed a luteinization of the granulosa cells.

Estrogenic changes were the rule. Some of these as illustrated in Figures 18 to 22, have already been mentioned. Figure 35 shows a marked atrophy of the ovary with hyaline masses marking the site of the ova.

Transplantation and biological behavior:—The males were slightly more susceptible than the females (39 per cent and 31 per cent). Gonadectomy seemed to increase the "takes" in males (47 per cent) and decrease them in females (24 per cent).

The latent periods fluctuated between 66 days and 8 days and the growth rates roughly paralleled the latent periods. These are extreme values: one tumor reached 16 × 12 mm. in 5 months and another 32 × 13 mm. in 2 weeks. The average growth rate of intrasplenic tumors was about 18 × 17 mm. in 1 month. The intraocular tumors filled the anterior chamber in 2 or 3 weeks. The intrahepatic tumors had a growth rate of about 21 × 16 mm. in 6 weeks.

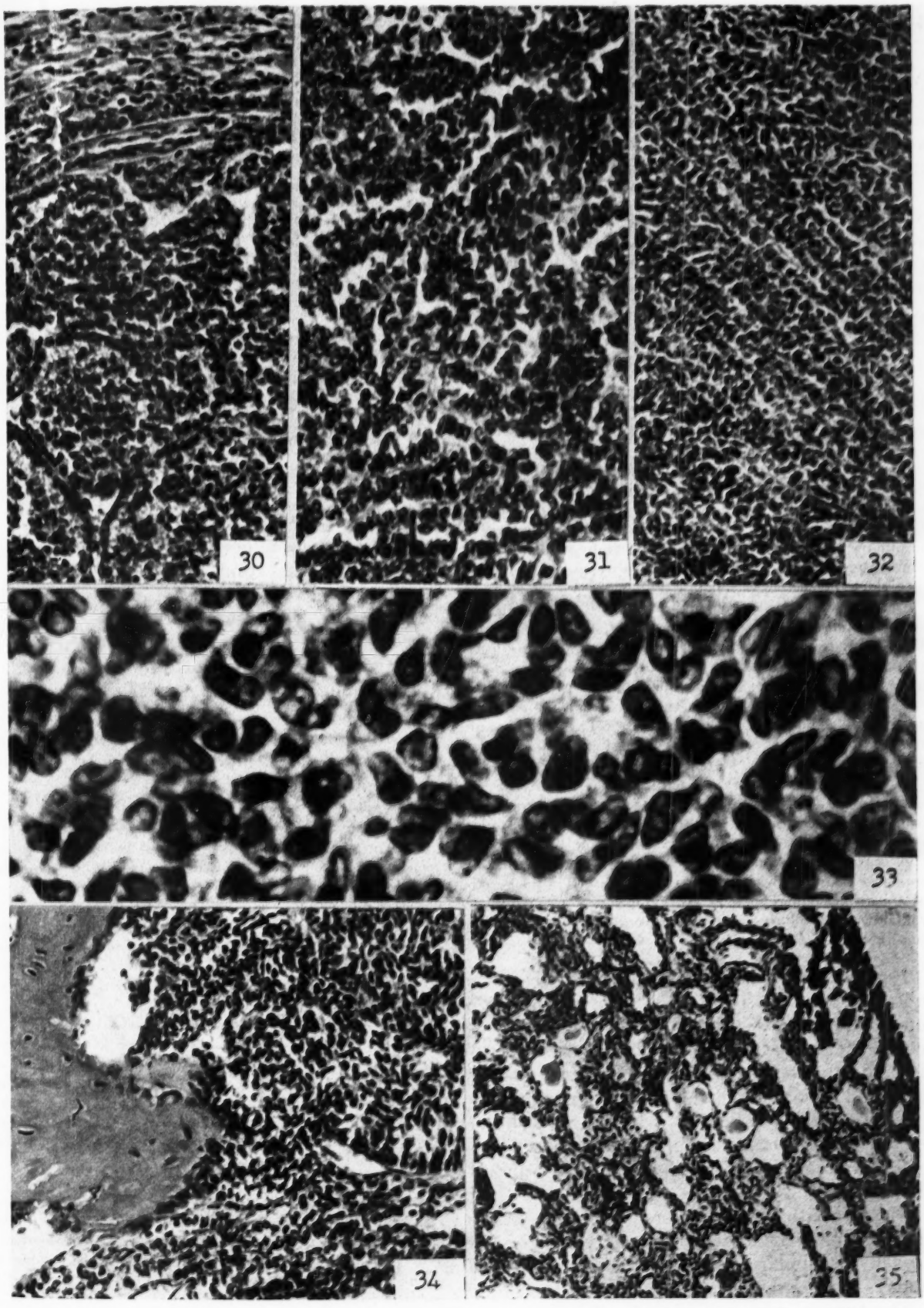
The tumors were malignant. Two were recorded to have penetrated the abdominal wall, 8 to have lung metastases (one from an intrahepatic, the others from subcutaneous tumors). In 27 mice, liver metastases were seen, all of which had come from splenic grafts. In 18, abdominal hemorrhages were seen, 11 of which had intrasplenic grafts and 3 intrahepatic tumors.

In 4 mice, the tumor particles were injected intrasplenically but at death no tumors were found in the spleen, instead, large tumor masses were present in the liver.

Estrogenic effect was recorded in 19 per cent of the 102 positive normal females and in 32 per cent of positive gonadectomized females; in 31 per cent of normal males and 6 per cent of gonadectomized males. The intrasplenic tumors had estrogenic changes only in the presence of perisplenic adhesions or liver metastases with few exceptions, while mice with splenic tumors usually had congestive changes.

Congestive changes were noted in 25 per cent of the positive normal females, in 17 per cent of 41 gonadectomized females and in 23 of the normal males and 19 per cent of gonadectomized males. Intraocular tumors did not have any secondary manifestations.

The following routes of transplantation proved more successful than the conventional subcutaneous route. Intraocular grafts gave 89 per cent takes in females, 45 per cent in males. Intrahepatic



FIGS. 30 to 35.—Strain V.

FIG. 30.—Pulmonary metastasis reproducing the folliculoid pattern. Third subpassage. × 300.

FIG. 31.—Solid, poorly differentiated growth but still having the characteristic granulosa pattern. Third subpassage. × 400.

FIG. 32.—Formation of rows, "moire-silk" pattern. First subpassage. × 250.

FIG. 33.—Solid growth; nuclear pattern resembles that of the germinal epithelium and does not show the chromatin arrangement of typical granulosa cells. Third subpassage. × 850.

FIG. 34.—Bone formation in the stroma. Third subpassage. × 250.

FIG. 35.—Atrophy of the ovary with hyaline remnants of the ova. Fifth subpassage. × 150.

and intrasplenic routes (55 to 78 per cent, Table 1). Very recently intramuscular grafts made in association with Dr. R. G. Gottschalk have yielded a high per cent of takes. This is the oldest strain carried in our laboratory. Now after 4 years of passages it grows rapidly and its appearance in sections is that of an anaplastic carcinoma, in places suggestive of reticulum-cell sarcoma. It grows rapidly, reaching 1 to 2 cm. in about 2 weeks, yet it produces both estrogenic and congestive changes.

Strain VI: Granulosa, Adenoma, Fibrosarcoma Lines

The original animal was irradiated with 175 r when 46 days old. Seventeen months later a yellow-grey firm spherical tumor 6 mm. in diameter was found at the site of one ovary. This was composed of closely packed germinal epithelial tubules; the lining epithelium had a large lightly stained nucleus with very fine chromatin granules and one or two nucleoli (Figs. 36 and 38). This type of nucleus is characteristic of the nuclei of active germinal epithelium seen in the mouse embryo. Among the tubules, small clumps of granulosa cells were seen, which in some places formed small follicles (Fig. 39).

In the first passage, some recipients showed an increment of granulosa cells which formed cords and follicular structures (Fig. 37). Luteinization was observed in some granulosa cells (Fig. 36). Connective tissue increment was observed in some tumors. In the passages some recipients developed a massive pure granulosa cell tumor while the others had the complex tumors with mere increment in granulosa cells, fattening and luteinization of these cells, and a slight increment in "oat-shaped" cells.

In the 2 recipients of this first subpassage, a sarcoma developed in the stroma (Figs. 39 and 40). The connective tissue stroma was marked in the donor of one of these sarcomas and it is probable that this had an inapparent sarcoma. In the second subpassage, a granulosa cell line continued as a pure line. In some sections numerous variations were observed in the germinal epithelial tubules such as ramifying forms and papillary proliferations; the lining cells varied from tall to flat endothelium-like cells.

Transplantation and biological behavior:—The subcutaneous transplantation of granulosa cell tumors was unsuccessful in normal mice. Gonadectomy enhanced transplantability of both granulosa and tubular adenoma lines. The latter was unsuccessful in normal females and successful in only 8 per cent of normal males, while it gave 79 per cent to 100 per cent takes in gonadectomized animals. Irradiation also enhanced susceptibility (Table 1).

The incubation period averaged 83 days in the first passage. This increased to an average of 105 days in the tubular adenoma line and decreased to 20 days in the granulosa line.

The tumors gave no evidence of malignancy. Six of the 11 gonadectomized females showed hormonal changes; these had subcutaneous tubular adenomas overgrown by granulosa cells. Congestive changes were noted in 2 of 5 gonadectomized males with granulosa cells in the tumors.

Strain VII: Adenoma Type

The original animal was irradiated with 175 r when 35 days old. Seventeen months later a laparotomy was performed. A grey-yellow tumor replacing the left ovary was removed and implanted subcutaneously in the host and other animals. The uterus and breasts of the host were hyperplastic. Two months later the animal died, and a tumor was found at the site of implantation. This was a tubular adenoma with markedly proliferated stroma and fattened cells in the intertubular spaces as in Figure 41. The first passage gave tubular adenomas with an abundance of granulosa cells forming nest and tubular structures which in places resembled very closely testis tubules (Fig. 42). The granulosa cells showed fattening in many tumors (Fig. 41). In this passage only the subcutaneous tumor in the donor was of the granulosa type. Many cells underwent a "brown degeneration" which is due to deposits of some acid-fast lipoid (ceroid) in the cytoplasm (10).

In subsequent passages the granulosa cells were more abundant. They formed nests, cords, and follicular structures, many fattened and luteinized.

Transplantation and biological behavior:—The subcutaneous injections remarkably failed in 31 normal mice with one exception but were fairly successful in gonadectomized mice. The latent period ranged between 109 and 312 days. The growth rate was also slow, tumors reaching about 13 × 9 mm. in 17 months in the first and second subpassage.

Four gonadectomized females had secondary hormonal changes and in none were congestive changes observed.

Strain VIII: Predominantly Adenoma Type

The original animal received 175 r at 33 days of age. Seventeen months later the ovaries were yellow, 5 × 3 mm. each. The left ovary had a small cyst; the uterine horns were atrophic.

The first passage gave a tubular adenoma partly overgrown by granulosa cells. In subpassages the granulosa cell masses among the tubules differentiated into cords and follicles in some recipients while in others they showed fattening and degenerative changes. Some had areas of fibrosis.

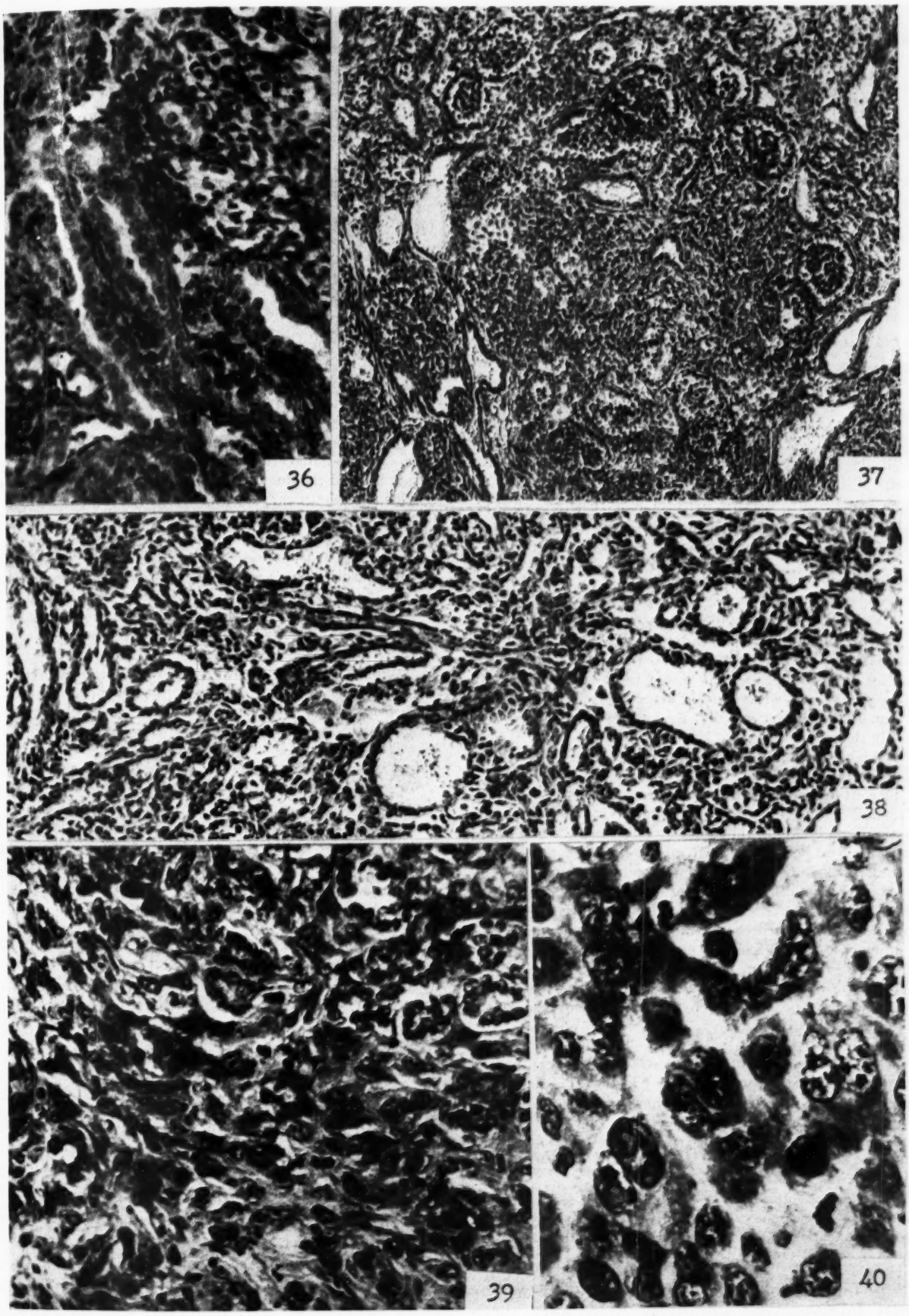
Transplantations were moderately successful (Table 1). The latent period was about 2 months. The growth rate was very slow, the tumors reaching about 10 × 3 mm. in 24 months in the first passage and 6 × 5 mm. in 19 months in the next passage. No invasion or metastasis was seen. Only one gonadectomized female showed hormonal changes. This had a tubular adenoma overgrown by granulosa cells. Congestive changes were absent.

Strain IX: Luteoma Type

A pure luteoma has been described (9). It is still being kept in the laboratory. Its hormonal effects are not as marked as years ago. A report on its ability to raise the blood volume very slightly and increase considerably the red cell level will be published (14).

Strain X: Adenoma-Granulosa Type

The original animal was irradiated with 175 r at 40 days of age. Seventeen months later a laparotomy was performed. The small tumor of the right ovary used for transfer was a tubular



FIGS. 36 to 40.—Strain VI.

FIG. 36.—Tubular adenoma with partially luteinized stroma cells. First subpassage. × 250.

FIG. 37.—Complex tumor with granulosa cells predominating. Anovular follicles. First subpassage. × 100.

FIG. 38.—A field, representative of this section, predominantly a tubular adenoma. Third subpassage. × 180.

FIG. 39.—The granulosa tumor is invaded by fibrosarcoma arising in the stroma. First subpassage. × 250.

FIG. 40.—Higher magnification of a sarcomatous area in the above tumor. Third subpassage. × 800.

adenoma with few granulosa follicles and many ceroid cells among them (Fig. 45). The uterus was hyperplastic.

In the first passage one recipient showed a preponderance of granulosa cells while others showed predominantly tubules of germinal epithelium type. The subpassage made from the latter gave tumors in which the granulosa cells formed the main structure. A second subpassage was unsuccessful. Figure 44 shows convincingly the origin of granulosa cells in tubules of the germinal epithelium.

Transplantation and biological behavior:—This was a poorly transplantable strain, successful in normal males only. After ovariectomy, however, subcutaneous transplantations were highly successful in females.

The long latent periods, 111 days in the first passage, 630 days in the subpassage, are noteworthy. The growth rate paralleled roughly the latent period. No invasiveness or metastasis was seen. Three gonadectomized females showed the hormonal changes but all three had adrenal cortical nodules (23). All three had tubular adenomas overgrown by granulosa cells. No congestive changes were seen.

Strain XI: Luteinizing Granulosa Type

The original animal was irradiated with 175 r when 47 days old, painted twice with methylcholanthrene, and killed 14 months after irradiation. The right ovary was replaced by a yellow tumor with grey and red areas measuring 25 × 12 × 10 mm. The left ovary was 2 × 3 mm. The uterine horns were slightly thickened.

The complex tumor was composed predominantly of lutein cells with finely vacuolated cytoplasm; among large islands of lutein cells an arborization of capillaries was seen. Germinal epithelial tubules and strands of fibroblasts were along the vessels. In the center of the lutein islands were masses of granulosa cells. The border between granulosa and luteoma areas was indistinct; in this zone of transition the granulosa cells acquired a larger and finely vacuolar cytoplasm, while retaining at first the characteristic coarse stippled nuclei. In the adjacent zone the cells acquired the characteristic vesicular nucleus of the luteoma cell with finely dispersed chromatin particles and one nucleolus.

The uterus and the vagina showed effects of luteinization: elongation of uterine horn with hyperplastic narrow glands, and mucification of vagina.

The small ovary of this host showed a tubular adenoma with many granulosa cells and masses of ceroid cells among them.

In the first passage most tumors had both granulosa and luteoma cells (Fig. 47). In subpassages some of the tumors were of pure granulosa cell type, others showed exclusively lutein cells; in others granulosa cells were "oat-shaped" and cytoplasm scanty (Fig. 46). They exhibited ability to form follicular structures.

In the second subpassage a hemangioma developed in one of the recipients which is still being carried as an independent line (1). Mixed types of tumors also persisted in some of the subpassages.

Transplantation and biological behavior:—The mixed luteoma and granulosa lines were more readily transplantable in males than in females. The latent period increased from 62 days in the first passage to 167 days in the subpassage and later declined slightly. The growth rate paralleled roughly the latent period. Two tumors penetrated into the abdominal cavity. Ten of 20 normal females and 6 of 29 normal males showed hormonal changes. Only the original animal had the secondary changes of both luteoma and ganulosa cells, the others showed only the lutein cell influence such as adrenal cortical atrophy and obesity (9). The pure granulosa cell tumors did not show hormonal changes.

Strain XIII: Adenoma, Granulosa, Fibrosarcoma Lines

The original animal received 175 r when 40 days old and was painted twice with methylcholanthrene (7). When killed 16 months later a 1 cm. tumor replaced the right ovary; the left ovary was atrophic. The uterine horn was hyperplastic with a polypoid mass in one horn. The mammary gland showed hyperplasia of the ducts.

The small left ovary contained a tubular adenoma with intertubular granulosa cells that were fattened in some areas. There were also areas of luteinized and ceroid cells.

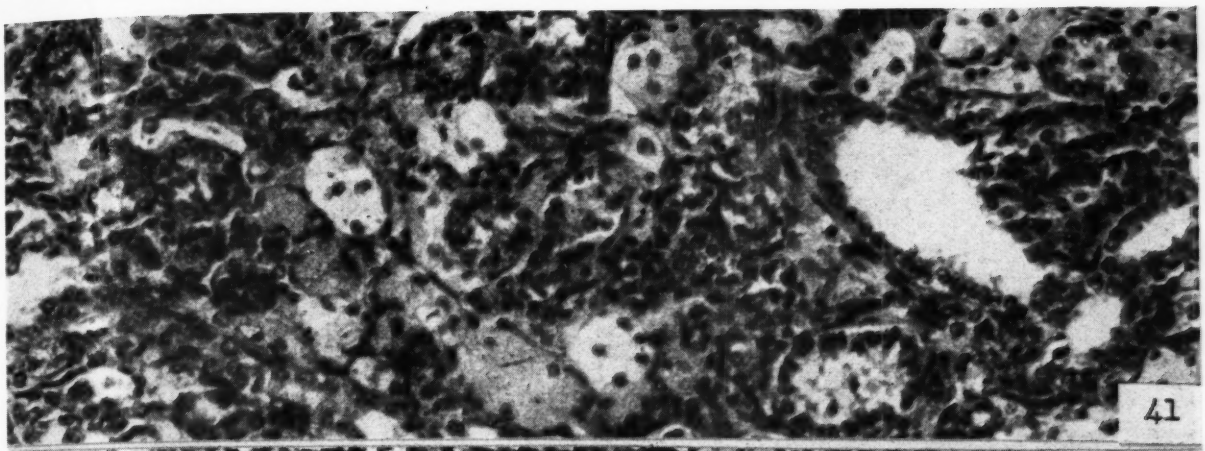
The right ovarian tumor had a follicular arrangement of granulosa cells with a basement membrane and dense papillary ingrowths (Fig. 48). In the first passage of this tumor the tendency to papillary formations was even more conspicuous. In some recipients there was cystic enlargement of the granulosa nodules, thus taking the shape of a mature follicle. In the first subpassage such formations closely resembling Graafian follicles were numerous. In some recipients, the granulosa cells formed a reticular structure, in others they closely resembled tubular adenoma, but retained the coarse stippled nuclei of granulosa cells. A metastatic well-differentiated folliculoma is shown in Figure 49.

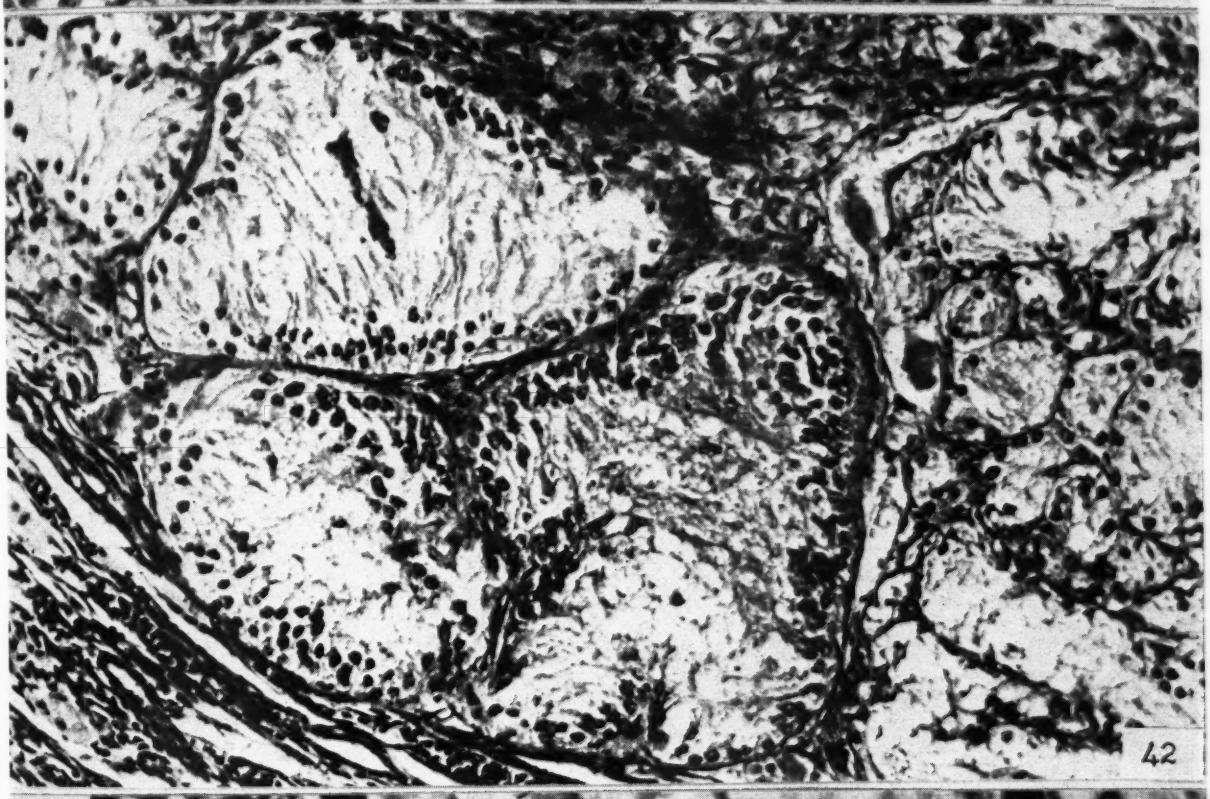
In still other cell recipients a sarcoma developed. Although they were discovered in seemingly pure granulosa cell tumors, we believe the latter contained sarcoma cells. At first the sarcoma had an irregular structure with large hyperchromatic nuclei and many giant and undifferentiated round cells (Fig. 50). In later passages the cells assumed the form of a more mature fibrosarcoma.

Transplantation and biological behavior:—There was no marked difference in susceptibility between males and females in the different lines (25 to 39 per cent). Intraocular implantations were unsuccessful. The latent period was 260 days in the first and 143 days in the subpassage. With the appearance of the sarcoma in the second subpassage the latent period dropped to 37 days and to 7 days in the third subpassage, in which the tumors were purely sarcomatous. The growth rate paralleled the latent period. The tumor measured about 8 × 7 mm. in 12 months in the first passage and 20 × 18 mm. in 1 month in the subpassage, and it did not change thereafter.

The tumors of specialized ovarian parenchyma showed but little invasiveness while the sarcomas had a great tendency to penetrate into the abdominal cavity.

Of 4 females that had tubular adenoma combined with granulosa tumors, one showed second-





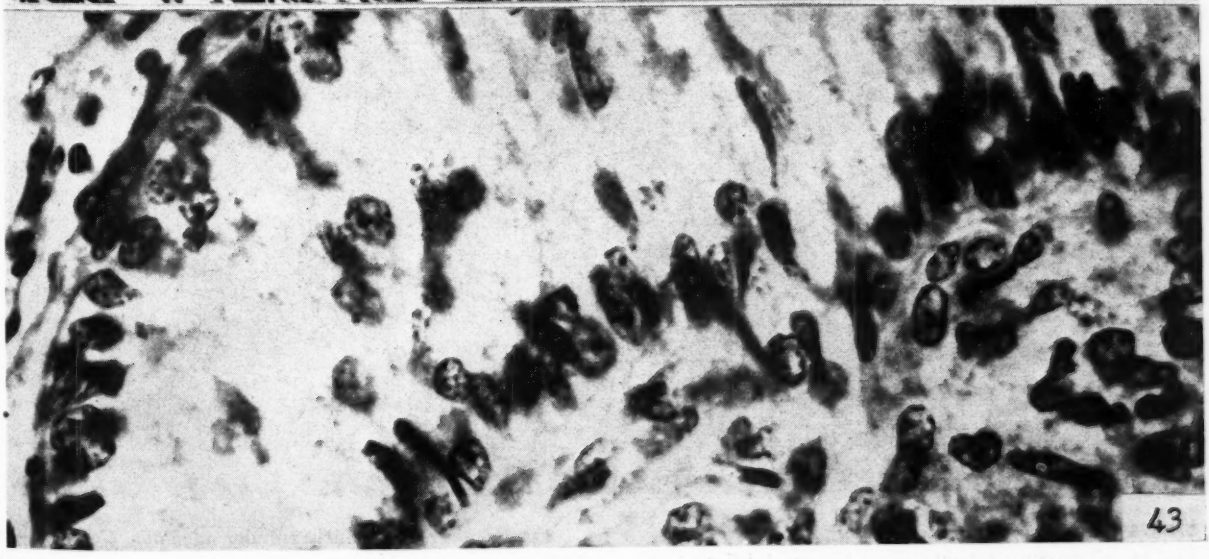
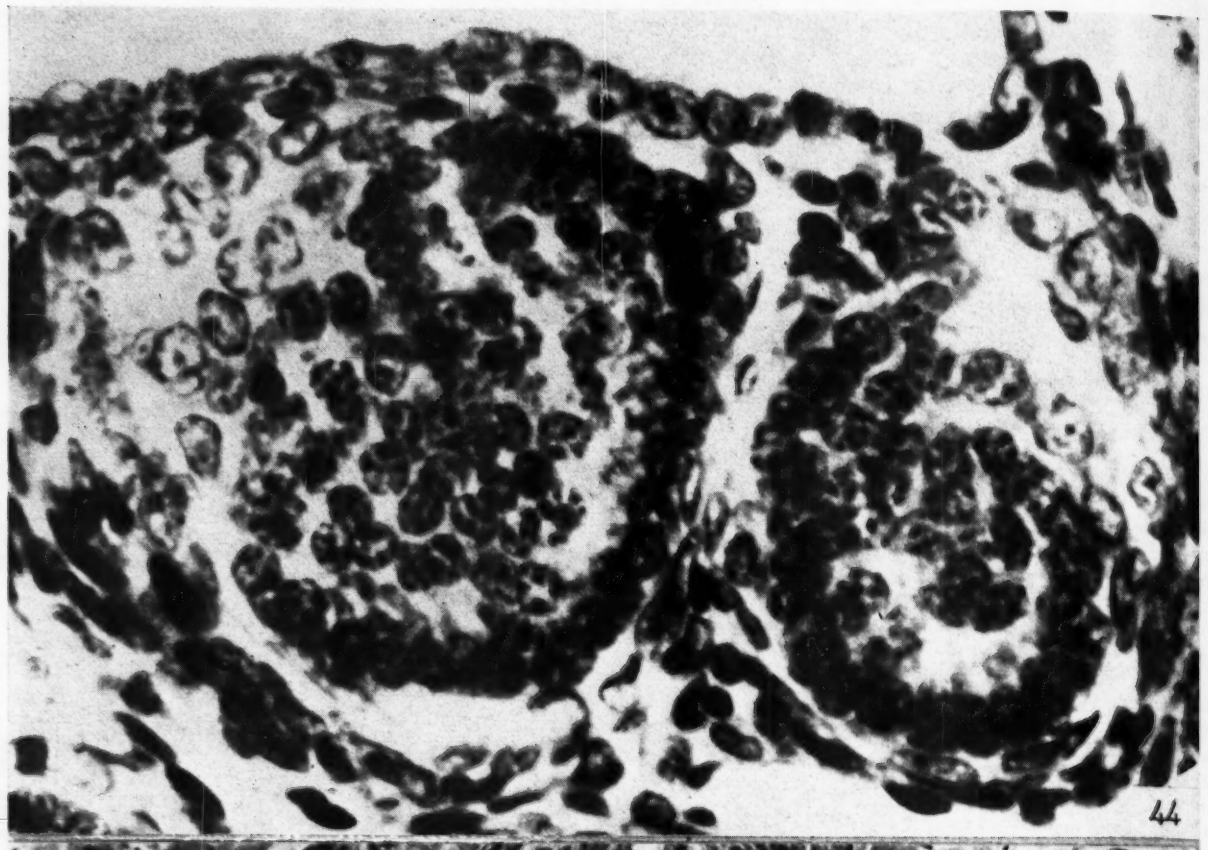
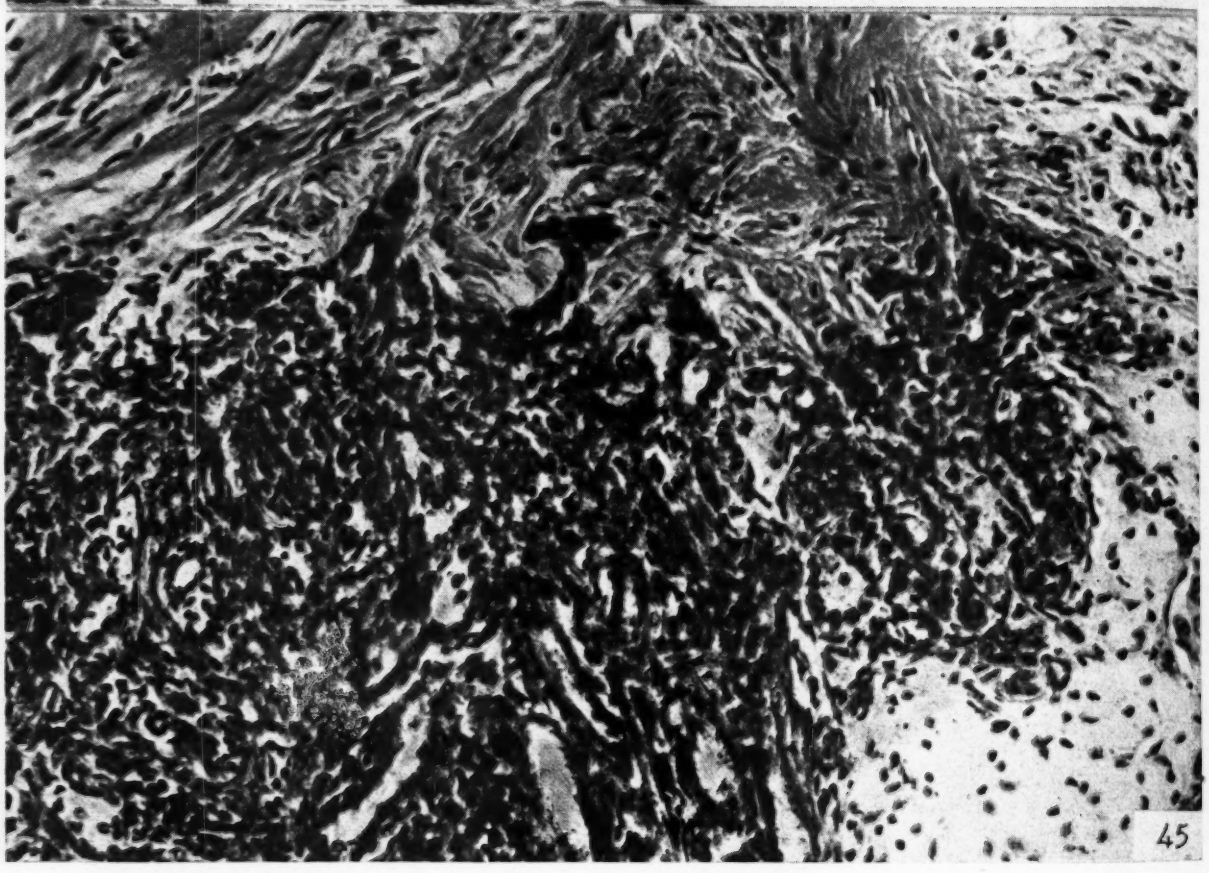


FIG. 41.—(Strain VII) Complex tumor. Few adenomatous tubules; small follicles of granulosa cells; many are "fattened," others changed into ceroid cells. First subpassage. × 250.

FIG. 42.—Tubular structures reminiscent of male (Wolffian) structures in a complex tumor of Strain VII. × 250.

FIG. 43.—Tubule formation in a granulosa tumor of Strain XIV. The cytoplasm is fibrillary, those of two rows of cells appear connected; the structure resembles testis tubules. First subpassage. × 660.

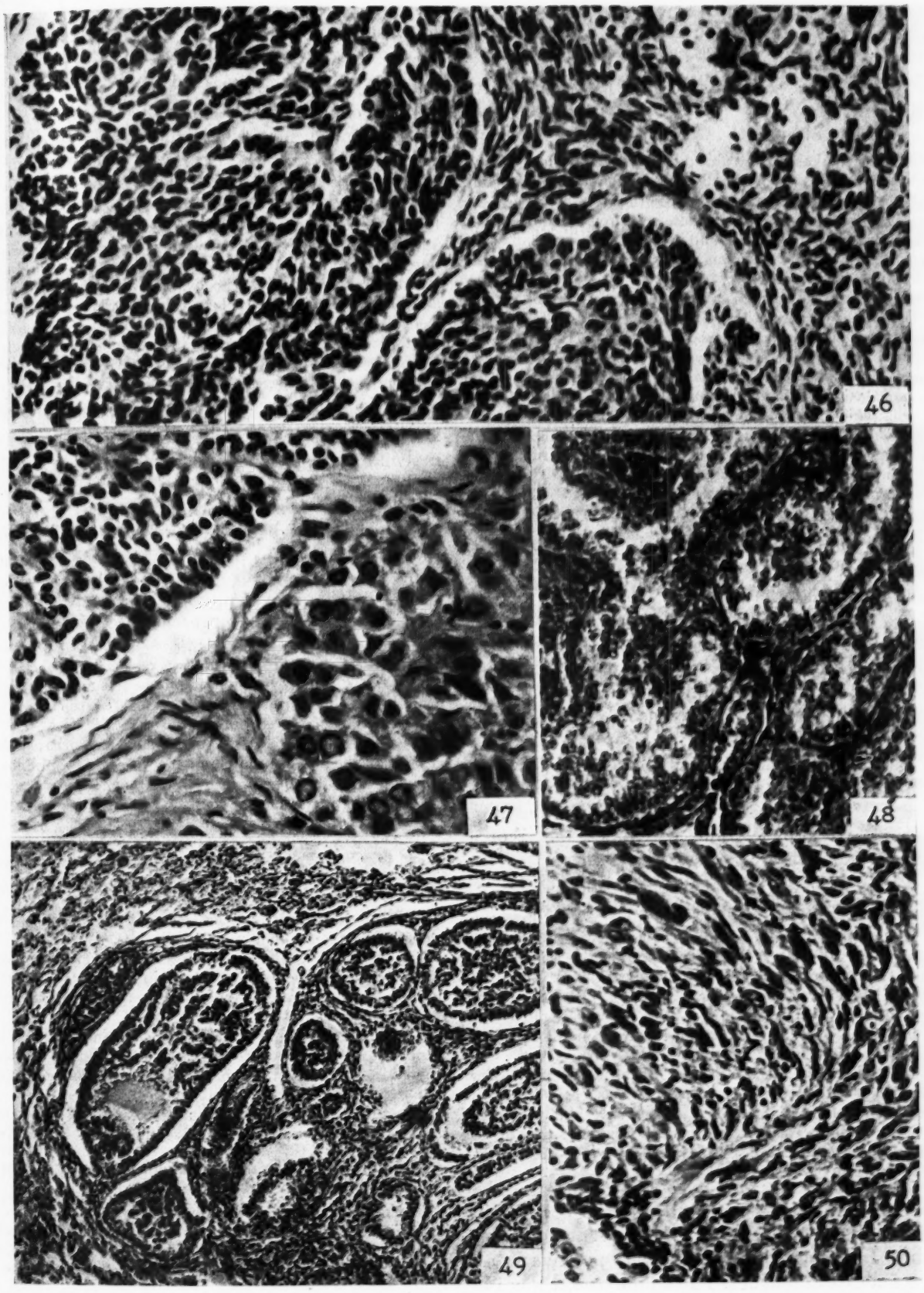




FIGS. 44 and 45.—Strain X.

FIG. 44.—Two convoluted tubules. Apparent differentiation of germinal epithelial tubules into granulosa follicle, both within the same basal membrane. First subpassage. × 850.

FIG. 45.—Predominantly tubular adenoma. Ceroid cells in right lower corner. The muscular wall of the uterine horn on the upper part of the field is invaded by the adenoma. Original tumor. × 200.



FIGS. 46 and 47.—Strain XI.

FIGS. 48 to 50.—Strain XIII.

FIG. 46.—Granulosa tumor with "oat-shaped" cells. These resemble those of the stroma but unlike the latter tend to form circumscribed areas some of which are reminiscent of follicles; the stroma cells form bundles but no circumscribed masses. First subpassage. × 200.

FIG. 47.—Junction line between large areas of well-differentiated luteoma and granulosa growths. Second subpassage. × 250.

FIG. 48.—Well-differentiated follicular growth of the original tumor XIII. × 250.

FIG. 49.—Pulmonary metastasis with reproduction of the follicular pattern. First subpassage. × 100.

FIG. 50.—An undifferentiated sarcoma in the third passage of a granulosa line. × 250.

ary hormonal changes; none had congestive changes.

Strain XIV: Granulosa Type

The original animal was irradiated with 87 r when 47 days old. When killed 29 months later a 12 mm. sized grey-pink spherical tumor was found to replace the right ovary and the left ovary was occupied by a yellow-grey growth 6 mm. across. The uterus was 4 mm. thick and cystic. The animal had also a generalized myeloid leukemia. The breast was hyperplastic and of lactating type.

In the first passage pure granulosa cell tumors were obtained with few unusual formations. The cells formed undifferentiated masses, follicular and occasionally tubular structures. The latter were lined with a single layer of cylindrical cells; some of these cells had a tall cytoplasm with a fibrillary structure toward the lumen (Fig. 43). Some tumors showed an abundant stroma which in some areas contained an intercellular substance with characteristics of mucin. In some sections the theca externa type of cells were numerous. In the second subpassage the same forms persisted and in addition "moire-silk" pattern was seen in some masses. These forms persisted in the subsequent passages one or the other structure being predominant.

Transplantation and biological behavior:—Gonadectomy increased transplantability. No significant difference was observed between normal females and males. The latent periods showed but slight differences. The growth rate fluctuated slightly, the tumor reaching about 22 × 14 mm. in $1\frac{1}{2}$ months. In one line the growth rate tended to increase; in another to decrease. No invasion or metastasis were observed.

Estrogenic changes were recorded in 7 of 10 gonadectomized, and in all 5 normal females, and in 8 of 9 normal males. One of 5 normal females, and 3 of 10 gonadectomized females, and 2 of 9 normal males showed congestive changes.

Strain XV: Predominantly Tubular Adenoma Type

The original animal received 175 r when 40 days old and was killed 23 months later. She had bilateral ovarian tumors and the pituitary was about 10 times the normal size. The right ovarian tumor was composed of germinal epithelial tubules containing papillary proliferations with a moderate number of granulosa cells. In the left ovarian tumor granulosa cells had overgrown the tubules.

In the first passage the derivatives of the left ovarian tumor resembled the original tumor. One tumor was locally invasive. Derivatives of the right ovarian tumor were granulosa cells with few tubules. The intrasplenic tumors had the same histological appearance as the subcutaneous tumors.

Transplantation and biological behavior:—The subcutaneous injections were more successful in normal males (17 per cent) than in females (4 per cent). Intrasplenic injections were positive in all of 4 males and females. The latent period was 144 days in the first passage and 229 in the subpassage. The growth rate was constant at about 14 × 11 mm. in 4 months. The tumors were not invasive and did not metastasize. Only one of the subcutaneously injected normal females with granulosa tumor showed hormonal manifestations. No congestive changes were seen. The intrasplenic tumors showed neither hormonal nor congestive changes.

Strain XVI: Adenoma Type

The original animal received 350 r when 40 days old. Twenty-three months later a laparotomy was performed and a 25 × 18 mm. sized yellow tumor replacing the right ovary was removed. The uterine horn was normal. When killed the animal had several hepatomas. The ovarian tumor was a tubular adenoma of germinal epithelium type. Strands of similar cells were also seen along some tubules with suggestive changes into granulosa type. In some areas granulosa cells formed follicles. Large cysts full of blood were present. In the first passage the granulosa cells overgrew the tubular adenoma.

Transplantation and biological behavior:—The implantations were moderately successful in normal males (16 per cent) and unsuccessful in females. The latent period was 51 days in the first passage and 73 days in the subpassage. The tumors were not invasive or metastasizing. In the first subpassage one of 4 positive males had both hormonal and congestive changes.

Strain XVII: Adenoma and Luteoma Lines

The original animal received 87 r when 33 days old. Twenty-nine months later the right ovary was replaced by a spongy tumor 2.5 × 2 × 1.8 cm. in size, and the left ovary by a grey firm tumor. The uterine horns were elongated, thickened, and cystic. Both tumors were transferred separately.

The left ovary had a tubular adenoma with papillary growths in the lumina and many cells with fine stippled nuclei among the tubules. The right ovarian tumor was composed of lutein cells with extremely dilated capillaries. The uterine horn had long and tortuous glands with scanty stroma.

In passages the tumors originating from the left ovary retained the original structure. The papillary growths and granulosa cells were slightly more numerous in some tumors; in others the granulosa cells were fattened.

The tumors originating from the right ovarian tumor were exclusively luteomas rich in mast cells.

The tubular adenoma line of this strain had a fair ratio of takes in males (26 per cent) and none in females. The luteoma line could also be transplanted with greater success in males than in females.

The latent period did not change significantly through the passages (about 113 to 125 days). The growth rate dropped from 22 × 12.7 mm. in 7 months in the first passage, to 5 × 5 mm. in 9 months in the first subpassage.

The tumors did not show any invasiveness and had no metastasis. No hormonal or congestive changes were observed.

Strain XVIII: Adenoma, Hemangioma, Reticulum Cell Sarcoma Lines

The original animal was irradiated with 350 r at 33 days of age and killed 17 months later. The right ovary was replaced by a yellow tumor 10 × 5 mm. which was used for transfer. The part sectioned showed a tubular adenoma with granulosa cells forming masses and cords of ceroid cells among them. A growth at the site of the left ovary was composed mainly of reticulum cells.

The first passage gave a reticulum cell sarcoma in one recipient and a tubular adenoma in the other. In the latter granulosa cells among the tubules formed follicles. A third tumor in this passage was a hemangioma.

Transplantation and biological behavior:—Transplantations were fairly successful only in ovariectomized mice. The latent period decreased from 204 days in the first passage to 94 days in the subpassage. Several tumors regressed in the subpassages. No invasion or metastases were noted. One of the 3 gonadectomized females with an adenoma rich in granulosa cells showed estrogenic changes; none had congestive changes.

DISCUSSION

Multiplicity of ovarian neoplasms induced by x-rays:—The first fact that emerges from these studies is the multiplicity of neoplasms that develop in the irradiated ovaries and the constancy with which they develop (7). Although serial sections were not made and the bulk of the tumors was often used for transfers, there is ample evidence indicating that most ovarian growths are complex containing at least three elements: tubular adenoma, granulosa, and luteoma of which sooner or later one type tends to overgrow the other. Using grossly separable pure fragments for transfer these neoplasms can be established as pure types.

In addition, sarcomas and endotheliomas may develop in the stroma (1). Many angiomas have doubtless been dismissed as mere telangiectasias because our interest was focused on hormone-producing cells.

Thus, five types of neoplasms seem to develop in x-rayed ovaries: 1) Tubular adenoma, a product of the germinal epithelium; 2) Granulosa cell tumors which we believe to be their derivatives; 3) Luteomas, the derivatives of granulosa cells; 4) Sarcomas; and 5) Angiomas and endotheliomas. Transformation of 1) into 2) and of 2) into 3), once established, seems irreversible.

Transplantations as performed require masses of cells and are influenced by so many variables that they do not directly indicate which of the cell masses transplanted are neoplastic and if so of what grade, conditionally neoplastic or normal. But those that were transplanted in series and grew progressively in normal hosts can be regarded as neoplastic.

Origin and interrelationship of the different induced neoplasms:—After a few passages the granulosa cell tumors and luteomas became pure lines and did not change in the course of many subpassages. In morphological appearance the granulosa cell tumors manifested about all variations described in human tumors (3, 22). The various strains possessed individual features as is the rule with neoplasms. The luteomas on the contrary were monotonously alike exhibiting only minor morphologic variations. They were characteristically rich in mast cells (2).

The tubular adenomas resulted from downgrowth of germinal epithelium and there were many intermediate forms between germinal epithelium and tubule formations. Morphological observations suggest that the germinal epithelial cells lining the tubules and cell masses among them can turn into granulosa cells. Consequently tubular adenomas are frequently overgrown by functioning granulosa cells. Nevertheless, tubular adenomas were transplantable as such and none carried in subpassages ended up as a pure, readily graftable granulosa tumor. This requires clarification.

No morphological evidence has been encountered, suggesting that the granulosa or lutein cells originate from the ovarian stroma cell (ovariocyte). In the irradiated ovaries, the ovarian stroma does not seem to give rise to secreting neoplasms. The character of the theca cell and the concept of embryonal multipotent character of the ovarian stroma cell requires reconsideration. In the histogenesis of ovarian tumors they appear to play a minor role, but the subject is highly controversial (13, 18, 21, 22).

The transformation of one cell type into another with acquisition of hormone production (germinal epithelium into granulosa) or change of type of hormone produced (progestin in place of estrogen) is a profound modification that appears irreversible, thus differing from common metaplasia. While transitional forms are abundant in the material here analyzed, once a tumor differentiated it remained true to type.

The lutein cell transformation is frequently confused with mere deposition of fatty material in the cytoplasm of granulosa cells also called luteinization. Granulosa tumors so affected are more correctly named "folliculome lipidique." The nuclei of these cells have the typical granulosa pattern and their cytoplasms are not those of acidophilic, sudan negative lutein cells. The cytogenetics and histochemistry of this transformation deserves further study. Evidence strongly suggestive of transformation of perhaps already neoplastic granulosa cells into lutein cells was seen only in Strain XI.

All strains that began as tubular adenomas were sooner or later overgrown by granulosa cells. Although the tubules may have been subjected to pressure by the surrounding granulosa cells they showed no degenerative changes. It is possible that undifferentiated stromal ovariocytes have differentiated into granulosa cells or the granulosa cells may have overgrown the tubular epithelium, or

the tubular cells may have been transformed into granulosa cells. Actually there were masses and anovular follicles of granulosa cells between the epithelial cells within the basement membrane of the tubules as illustrated. Some lining epithelial cells of such tubules were of the granulosa type; others were of germinal epithelium type. This could be explained as invasion of the tubules by granulosa cells. However cells of the granulosa type could be found around small germinal epithelium formations outside the ovarian tissue, where an ovarian origin is unlikely. Conversion of the tubular adenoma into a granulosa cell tumor was also noted by Li and Gardner (18). This is a field in which morphologists studying the same situations have arrived at opposing view-points, and clarification awaits further work with newer techniques.

Fibrosarcomas originated in one granulosa and in two mixed (tubular adenoma and granulosa) strains. Two of the fibrosarcomas appeared in the second generation and one in the sixth generation. Hemangiomas occurred in two granulosa, one luteoma, and one in a complex strain. One of the hemangiomas appeared in the first passage, the others in the fifth, third, and second passages. After their appearance these tumors rapidly replaced the original structures. Two hemangioma strains became increasingly malignant and anaplastic (1). The sarcomas on the other hand, although equally malignant, developed more mature features.

The available evidence suggests that these tumors were already present in the original growth in minute foci. Going back to a section of the original tumor we actually saw them in one case. We believe that these "secondary" tumors originated from the stroma of irradiated ovaries and that the respective cells carried with them potency of neoplastic proliferations through a few subpassages of the carrier tumors as Cohnheim nests, until favorable conditions enabled them to form neoplasms of their own. The possibility that stroma cells of transplanted tumors may undergo malignant transformation should be considered.

Malignancy of induced tumors:—The x-ray induced tumors have been compared with those that arise in grafts of ovaries in spleen of castrated mice (4, 12, 11, 16). Both seem to contain cells with a wide range in character varying from those non-neoplastic to those highly malignant. On the basis of transplantability to normal hosts most x-ray induced growths appear to be neoplastic in contrast to those induced by the Biskind procedure, most of which appear to be mere hyperplasias (10).

Many granulosa tumors and some luteomas manifested malignant properties such as invasiveness and ability to metastasize. Metastasizing granulosa cell tumors are rare in man. Some mouse strains are more likely to metastasize than others. Some had metastases in the original animal while others manifested this potency only after a few passages. Pre-irradiation and gonadectomy of the host did not seem to influence the formation of metastases. In microscopic appearance the metastases were similar to the original tumor. The sites of metastases of the subcutaneous tumors were (in order of frequency): lung, liver, kidney, and lymph nodes. The latter were not often examined microscopically.

Metastases of intrasplenic tumors to the liver were a common finding. In several instances hepatic metastases appeared without a detectable primary growth in the spleen. The impression is gained that granulosa tumors metastasize by way of the blood stream rather than the lymphatics. Although all but one of the metastasizing tumors were large, there is no direct evidence suggesting that metastases are proportional to the size of the tumor; neither do they seem to depend on the growth rate or the duration of the tumors.

Factors influencing the success of transplantation:—Difficulties at transplantation may be due in part to genetic, in part to other factors that deserve a thorough analysis since they may disclose forces that influence the genesis and the growth of spontaneous tumors in general. Although both donors and recipients were first generation hybrids of Rf/Ak mice, we are uncertain as to the genetic homogeneity of donors and recipients. The experiments lasted many years during which several sublines were established and donors and recipients came from varied sublines. The genetic composition of both tumors and animals often change with successive generations; in spite of this, several important factors were identified.

Sex and hormonal influence.—Transplantation of granulosa cell tumors is slightly more successful in males than in females. The sum of transplantation results of seven granulosa strains is as follows:

		Normal		Gonadectomized			Pre-irradiated
Recipients		Subcut.	Intrasplenic	Subcut.	Intrasplenic	Intrahepatic	Subcut.
Males	No. of mice	759	19	99	37	11	78
	Takes, per cent	42	63	48	54	64	38
Females	No. of mice	694	19	163	26	17	120
	Takes, per cent	35	32	34	54	71	39

More recent transplantations with Strain V made in association with Dr. R. G. Gottschalk have confirmed this trend.

The following summary of transplantation of three luteoma strains shows a trend to some extent the reverse of that of the granulosa tumors:

Recipients		Normal		Gonadectomized		Pre-irradiated
		Subcut.	Intra-splenic	Subcut.	Intra-splenic	Subcut.
Males	No. of mice	205	2	23	4	6
	Takes, per cent	44	50	39	25	33
Females	No. of mice	116	2	18	9	8
	Takes, per cent	50	100	67	25	75

An unexpected observation is the marked increase in the per cent of takes in grafting tubular adenomas in gonadectomized mice as shown by the following tabulation of nine strains in which the cells were predominantly of this type:

Recipients		Subcutaneous grafts		
		Normal	Gonadectomized	Irradiated
Males	No. of mice	157	21	12
	Takes, per cent	18.5	48	25
Females	No. of mice	126	63	18
	Takes, per cent	5.6	54	39

This is a combined tabulation of nine strains (Table 1). Several of these strains could be grafted in males only and gonadectomy greatly raised the per cent of takes particularly in females (Table 1). This observation suggests that these adenomas may be inhibited by hormones of the gonads, particularly in females, or stimulated by excessive amount of pituitary gonadotrophins. This working hypothesis brings the adenomatous tumors of the ovary in the group of those whose endocrine genesis and control may be possible.

The endothelioma strain that resembled a (non-secreting) chorio-epithelioma likewise grew better in gonadectomized mice, while a typical endothelioma did not:

Endothelioma strain		Subcutaneous grafts			Intrasplenic
		Normal	Gonadect.	Pre-irrad.	Gonadect.
IV. Chorio-epith.-like	No. of mice	104	21	9	9
	Takes, per cent	31	71	33	100
XI. Hemangioma type tumor	No. of mice	108		8	14
	Takes, per cent	31		75	0

It is possible though unlikely that strain IV was actually a chorio-epithelioma and is therefore influenced by the endocrine state of the host. It is of practical importance to know factors that markedly influence the per cent of takes, such as gonadectomy, irradiation, sex, and sites of implantation. But for theoretical interpretation more data are needed such as the effect of hypophysectomy and of hypophyseal and other hormones, alone and combined, on the transplantability of ovarian tumors. Furthermore, data are needed on the specific relation of these factors to tumors of different types.

Latent period and the growth rate:—Enormous variations were encountered. The latent periods were longest with tubular adenomas, averaging 207 days, while those of granulosa cell tumors were 80 days, and of luteomas 68 days. The longest latent period was also encountered among the tubular adenomas (about 2 years) while that in granulosa lines was 260 days and in luteoma lines 167 days. The growth rates were also greater in the granulosa and luteoma tumors than in tubular adenoma.

Hormonal activity.—The observations concerning hormonal activity were merely incidental to this work and require a special study. All granulosa tumors and luteomas that have been studied for some time have given evidence of stimulation by estrogens or progestins respectively, while the tubular adenomas appear inactive.

A unique feature of the granulosa tumors is their ability to produce congestive changes with rise in the blood volume. This is due to a hypothetical substance, now named plethorin, which is directly or indirectly related to granulosa cells.

The question arises whether or not the two types of activities of granulosa cell tumors (estrogenic and congestive) are bound to the same substance. Estrogenic activity of the tumors begins earlier than the congestive change (Table 2). The granulosa cells that have overgrown the tubular adenomas had only estrogenic activity. The two can occur independently (Table 3). The estrogenic changes seem to come to a standstill when the tu-

TABLE 2

The Relation Between Tumor Size and Internal Secretory Activity of Granulosa Tumors

Strain		Tumor size 1+*	2+	3+	4+
I	No. studied	9	10	21	58
	No. with estrogen production	0	0	6	21
	No. with plethorin production	1	2	11	40
II	No. studied	1	10	12	24
	No. with estrogen production	0	2	4	10
	No. with plethorin production	0	2	2	11
III	No. studied	1	3	20	37
	No. with estrogen production	0	2	13	21
	No. with plethorin production	0	0	17	29
IV	No. studied	12	12	6	7
	No. with estrogen production	1	0	0	2
	No. with plethorin production	0	0	0	0
V	No. studied	13	15	22	45
	No. with estrogen production	1	6	12	24
	No. with plethorin production	0	4	10	24
VI	No. studied	0	4	0	0
	No. with estrogen production	0	1	0	0
	No. with plethorin production	0	2	0	0
VIII	No. studied	5	0	2	0
	No. with estrogen production	0	0	0	0
	No. with plethorin production	0	0	0	0
XIV	No. studied	0	8	7	2
	No. with estrogen production	0	7	7	1
	No. with plethorin production	0	1	3	1
Total no. studied		41	62	90	173
No. positive with estrogen production		2	18	42	79
No. positive with plenthorin production		1	11	43	105
Per cent estrogen production		4.8	29.0	46.6	45.7
Per cent plethorin production		2.4	17.7	47	60.7

* Tumor Size: 1+ = The two greater diameters less than 10 mm.
2+ = The two greater diameters each 10 to 20 mm.
3+ = One of the greater diameters is less, the other greater than 20 mm.
4+ = Both diameters greater than 20 mm.

The figures in Tables 2 and 3 include normal, gonadectomized and irradiated hosts. The organs studied for estrogenic effects were: vagina, uterus, ovary, submaxillary gland; testes and seminal vesicles. For localization of congestive changes see text.

Intrasplenic tumors are included in Table 2 but not in Table 3. Intraocular tumors are not included.

The two mice of Strain VI that showed congestive changes only, were among the five mice with granulosa tumors that have overgrown tubular adenomas, as was one mouse of Strain VII.

TABLE 3

Relation Between Estrogenic and Congestive Changes

Strain	Number studied	Number of cases: *Changes* Estrogenic only	Congestive only	Both	Neither
I	101	5	31	22	43
II	47	8	8	7	24
III	61	5	22	25	9
IV	63	12	5	24	42
V	100	17	14	38	31
VI	4	1	2	0	1
VII	1	1	0	0	0
XIII	7	0	0	0	7
XIV	22	14	0	6	2
Total	406	63	82	102	159
Per cent		15.5	20.2	23.1	36.7

mors reach about 2 cm. in diameter whereas the congestive changes progress. These findings suggest that the two types of activities are independent of each other.

Another observation pointing in this direction is that the estrogenic substance is inactivated in the liver, whereas the congestive substance does not seem to be affected by the liver:

SECRETORY ACTIVITY AFTER INTRASPLENIC GRAFTS

	With liver metastasis		No liver metastasis	
Granulosa strains	Estrogen	Plethorin	Estrogen	Plethorin
I	1/4	*2/4	0/3	2/3
II			1/2	*2/2
V	5/25	**5/25	4/34	**6/34
Total	6/29	***7/29	5/39	***10/39
Per cent	20.7	24.1	12.8	25.6

Fractions indicate number of positive mice over the number studied. Each asterisk designates an animal that also exhibited estrogenic stimulation.

The above data suggest that estrogen and plethorin are different substances. It has been suggested that plethorin originates in the liver (cf. 20).

It is known that the blood volume rise precedes that of recognizable congestive changes. Thus many more animals had an increased blood volume than indicated in the tables. This is probably true also for estrogenic stimulations. The precise relation of the two remains to be analyzed. However, there is ample evidence to conclude that plethorin is specifically related to granulosa cells.

Pathogenesis.—Hormonal imbalance has recently been stressed as the most important factor in the pathogenesis of ovarian tumors (12, 18) and the now classical Biskind procedure (4) and the many observations of Gardner and his associates (12), indicate that it doubtless is a major factor. However, there is no evidence indicating that the growths induced by the Biskind procedure and the nodules frequently found in endocrines are truly neoplastic, but some can turn into true cancers.

We are impressed as are Van Eck-Vermande and Freud (21) by the double effect of irradiation: an immediate which is destructive and a delayed, which is an anomaly in development, "comparable to mutations obtained by x-rays where an invisible immediate effect entails a new trend in later development" (21). The immediate effect is a disorganization of the ovary; this is followed by a disturbed regeneration; some cells stimulated by the hypophysis proliferate and secrete and these in turn influence other cells of this organ and the pituitary itself. The magnitude of the delayed x-ray effect on cells of the ovary, the role of disorganization, and of the varied hormonal influences, all basic problems of tumor genesis, remain to be analyzed.

SUMMARY AND CONCLUSIONS

Exposure of ovaries to x-rays causes the development of tumors of different sorts. Five types have been transplanted in series: granulosa tumors, luteomas, tubular adenomas, sarcomas, and angioendotheliomas.

The granulosa tumors occur in such a wide range of morphological forms and simulate so many different types of neoplasms that their identification on a morphological basis alone is often not possible.

Common to all granulosa growths is the ability to produce or initiate production of estrogens and plethorins (a substance that raises blood volume) although not all tumor-bearing hosts show effects of these substances.

Manifestations of estrogen and plethorin stimulations can occur independently, although both are related to activities of granulosa cells.

No evidence was found to indicate that any cell other than a variant of the granulosa cells secretes estrogens.

Well-developed granulosa tumors did not change into luteomas. After a few transplantations each strain had certain morphological features that remained true through numerous subpassages.

All transplantable luteomas studied produced secondary changes indicative of progestin production.

The tubular adenomas are derivatives of the germinal epithelium. Those transplanted were benign and of exceedingly slow growth. In the course of subpassages sooner or later most of them either changed into or were overgrown by granulosa cells.

Male hosts are more susceptible to grafts of tubular adenoma and of granulosa tumors than females. This sex influence does not seem to be due to a gonadal hormone since it is usually magnified by gonadectomy.

Spleen and liver are better soils for granulosa grafts than the subcutaneous tissue; the success of splenic grafts is not necessarily due to inactivation of estrogens in the liver.

In the genesis of these tumors two major forces

are postulated; direct and delayed x-ray effect, and a hormonal "imbalance." Both require a more precise analysis to unravel the complex sequence of events initiated by irradiation of ovaries. The exposure to x-rays may last only for seconds but the chain of events which follows covers the entire life span of the animal.

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ADDENDUM

Discussions at the American Association for Cancer Research meeting on April 16 and 17 disclosed that there are no established criteria to differentiate by bioassay, androgens from different organs (testiculoids, progestins, and corticoids). Masculinization of submaxillary gland is produced by testiculoids only according to unpublished observations of A. Kirschbaum and M. J. Frantz.

In the present study no major role is attributed to interstitial cells: the luteomas are related to granulosa cells and none of the hormone-secreting tumors are derived from interstitial cells. These observations are contrary to those reported by others and should lead to further study but not to generalizations.

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Studies in Hodgkin's Syndrome

IX. The Association of "Viral" Hepatitis and Hodgkin's Disease

(A Preliminary Report)

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Viral hepatitis has aroused clinical interest and scientific curiosity because of the associated high mortality in old and in debilitated patients, the occurrence of irreversible liver damage in a small number of cases, the epidemic potentialities of the "infectious hepatitis" form of the disease and the unsuspected presence of either the "serum hepatitis" or the "infectious hepatitis" agent in a small but important number of individuals who donate their blood for parenteral administration to other humans.

The appearance of serum hepatitis (SH) in individuals who have received a parenteral inoculation of human serum containing the hepatitis agent, was first described by Lürmann in 1885 (11). Infectious hepatitis (IH), a contagious disease (3) which occurs sporadically as well as in epidemics, is transmitted to affected individuals by way of the gastro-intestinal tract and possibly by other routes such as the lung. Infectious hepatitis (IH) can also be acquired by parenteral inoculation of serum containing the IH virus. Criteria for differentiating these two types of hepatitis virus are based primarily on: (a) the longer incubation period of SH (2 to 6 months as compared with 2 to 6 weeks for IH), (b) the greater severity of SH, (c) the transmission of SH by parenteral inoculation only, (d) the absence of the SH agent in the feces and nasopharyngeal washings, (e) the protection afforded by prophylactic administration of pooled human gamma globulin in IH but not SH (4), (f) the failure of either disease to immunize against a subsequent infection due to the other (8, 13, 15), and (g) the more insidious and less febrile type of onset usually associated with SH (5).

The ameliorating effect of "catarrhal" jaundice on "a form of chronic joint disease in children" was described first by Still in 1897 (18). In 1932 improvement in rheumatoid (infectious) arthritis following cincophen-induced jaundice was described by Parsons and Harding (14) and by Grigg and Jacobsen (7) in 1933. Later, in 1933, Hench (9) described the temporary disappearance of or improvement in symptoms in the same disease syndrome following many varieties of jaundice including the obstructive types, presumably unrelated in etiology to "catarrhal" jaundice. Boros in 1937 (1) described the "dramatic" ameliorating effect of cincophen-induced jaundice on hay fever and asthma and Hertzler and Morlock and Alvarez, quoted by Hench (10), reported a similar effect on migraine in patients with jaundice, cirrhosis, and biliary tract disease. Hench (10) concludes that "hepatic damage" (of diverse etiology) "generally with but occasionally without jaundice" is responsible for the ameliorating effects observed and states that the anti-rheumatic and anti-allergic phenomena observed appear to be "group specific rather than disease specific." Numerous accounts of remissions induced by a variety of infectious diseases in patients with lymphomata and other neoplastic diseases have been published during the past eighty-three years (2).

The observations described in this preliminary report deal with clinical and laboratory changes which have been observed in three patients with Hodgkin's disease after the onset of viral hepatitis. Case 1 (V.H.) and case 2 (M.C.) have been followed for 5 and 6 years respectively after symptoms of hepatitis were observed. Case 3 (E. M. F.) died 4 days after the onset of jaundice.

Encouraged by an apparent improvement in cases 1 and 2 following viral hepatitis, the authors inoculated 21 Hodgkin's disease volunteers, cases 4 to 24, with a total of 35 samples of sera and tissue extracts containing the hepatitis virus. Clinical and laboratory observations in this latter group have been in progress for 6 to 12 months.

RESULTS

Case 1 (*V. H.*):—Hodgkin's disease was established by biopsy on 2–15–43 and, subsequent to the development of infectious hepatitis (IH) 7–12–44, the patient experienced a clinical and laboratory remission of 4 years duration (Chart 1):

Rapidly enlarging right and left supra-clavicular masses were observed in a 21 year old white male during February 1943. Biopsy study, reviewed by three pathologists, confirmed the clinical diagnosis of Hodgkin's disease. The patient was first seen by the Ohio State University Hodgkin's disease clinic during March 1943 and was given 1800 r over the right and 1800 r over the left cervical regions. The typical massive collar distribution of adenopathy disappeared following therapy. During April 1943 an enlarged node in the left axilla was treated with 1500 r and during June, one in the right axilla with 1800 r. The patient felt well until April 1944 when he incurred a severe back strain while unloading a truck. The diagnosis of herniated intervertebral disc was considered but not proved. On April 26, 1944, 3 mc. of radioactive phosphorus were given orally and several days later the patient complained of nausea, back pain, "stomach pain," anorexia, and postprandial pains. During June of 1944, 2000 r were given over the left groin with subsidence of adenopathy in that region. On July 12, 1944, the patient became jaundiced and reported for examination complaining of upper lumbar pain similar to that described during April, 1944. Fifteen hundred r were given over the upper lumbar region posteriorly. On July 26, because of severe jaundice and anemia the patient entered another hospital where he received

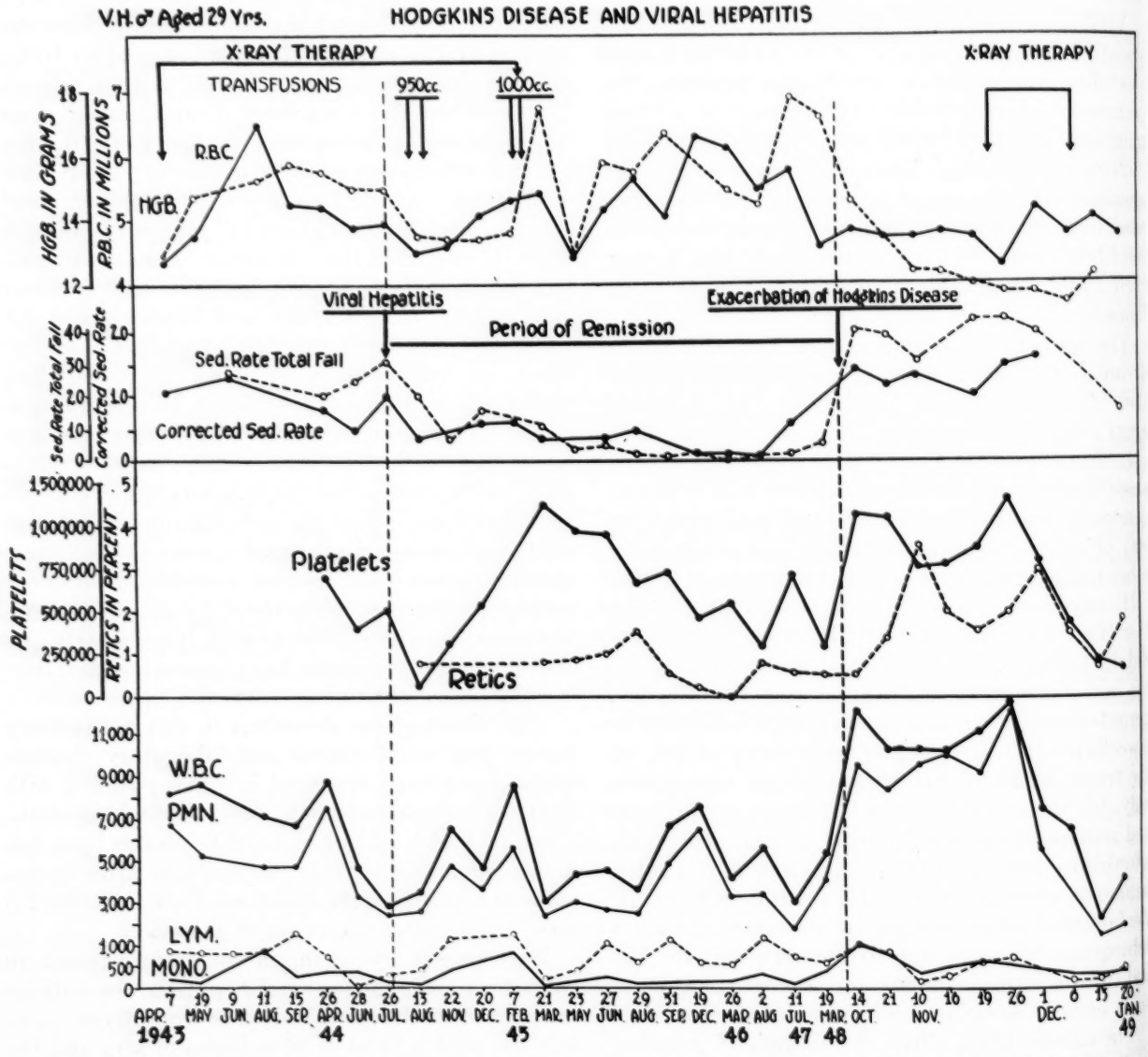


CHART 1.—Case 1. The day on which jaundice was observed clinically is recorded as the onset of viral hepatitis. The decrease in hemoglobin and red blood cells, the increase in sedimentation rate, the increase in reticulocytes, and the polymorphonuclear leukocytosis which accompanied the "exacerbation of Hodgkin's disease" are to be noted. Changes in sedimentation rate are more closely correlated with changes in the clinical status of the patient than are other hematologic findings.

transfusions and was kept at absolute bed rest from 7–26–44 to 9–13–44. An immediate and sustained return of appetite began following transfusions and bed rest. Improvement continued with the exception of complaints of upper lumbar pain and anorexia in December 1944. Fifteen hundred r were given both anteriorly and posteriorly over the upper lumbar region. It is probable that the "upper lumbar pain" complained of during December 1944 was not due to Hodgkin's disease. Occasional vomiting occurred after meals during February 1945 and shingles, anemia, and reddish-brown urine were noted during March 1945. A clinical diagnosis of viral hepatitis was made at that time. From July 1944 to November 1948 no symptoms which could be ascribed directly to Hodgkin's disease were noted. In November 1948 the patient described a feeling of "going downhill for the first time in 4 years" and complained of weakness, constipation, dysuria, and pain in the region of the right obturator foramen. Biopsy of a recently developed mass in this region revealed Hodgkin's granuloma. On rectal examination, digital pressure on the part of the mass within the pelvis produced movement of the part of the mass palpable through the skin over the obturator foramen posteriorly. A routine x-ray photograph of the chest indicated marked bilateral widening of the mediastinum of recent origin. The patient's description of the length of freedom from symptoms following hepatitis corresponds well with changes observed in the sedimentation rate (Chart 1).

Case 2 (*M. C.*):—After joining the Armed Services in 1942, M. C., a known case of Hodgkin's disease of five years duration, received a routine yellow fever vaccination and within approximately 21 days developed what appeared to be "viral hepatitis." Following the episode of jaundice of one weeks duration, a four year period ensued during which no clinical or laboratory evidence of Hodgkin's disease was elicited:

An inguinal lymph node biopsy study in 1937 in a 32 year old white male indicated the presence of Hodgkin's disease. During the period 1937 to January, 1942, the patient received "occasional courses" of x-ray therapy over the inguinal, axillary, and cervical areas with remissions lasting 6 to 12 months following each course. The estimated number of courses given during this period is 7.

Shortly after enlistment in the U.S. Army in December 1942, he developed general malaise, easy fatigability and weight loss presumably on the basis of Hodgkin's disease. X-ray therapy was not given since no evidence of adenopathy was found. He was given a tetanus inoculation and yellow fever vaccination at this time and states that jaundice developed accompanied by chills and fever approximately 3 weeks later. Chills and fever lasted 2 days and jaundice remained for 1 week. The patient experienced no further untoward symptoms in spite of rigorous military service and was discharged presumably in good health in November 1945 as a part of the routine demobilization of that year.

No further symptoms of Hodgkin's disease were observed until January 1947, 4 years after the onset of hepatitis and 5 years after the last x-ray treatment had been received. At this time he was found to have ascites and mediastinal adenopathy. A course of nitrogen mustard therapy was given followed by roentgen radiation, 1500 r to each of 4 ports over the anterior and posterior mediastinum and epigastrium. A remission occurred which lasted until September 1948 at which time examination revealed generalized adenopathy, hepatomegaly, and a left pleural effusion. A course of 42.5 mgm. of nitrogen mustard was followed by a remission and the patient again returned to normal activity. The patient's history includes a statement that remissions following x-ray and nitrogen mustard therapy were associated with "vague fatigue" whereas the 4 year remission following viral hepatitis was not.

Case 3 (*E. M. F.*):—A terminal case of Hodgkin's disease of 4 years duration elected to receive a parenteral injection of serum obtained from V. H. (case 1 above) and developed viral hepatitis 36 and died 40 days thereafter. Hepatitis in this case may have resulted from one of several transfusions received previous to and following the administration of V. H. (case 1) serum. Hemorrhages 1 mm. to 4 cm. in size within the Hodgkin's lesions observed at postmortem are described (Fig. 5).

A 40 year old white male noted enlarged axillary nodes during the fall of 1944 and subsequently developed cervical adenopathy, increased weakness, a 10 pound weight loss, and progressive dyspnea. A cervical lymph node biopsy study in May 1945 revealed Hodgkin's disease.

When seen in December 1945 at the Ohio State University Hodgkin's clinic, he had received no treatment and had generalized adenopathy and hepatomegaly. Another biopsy at this time confirmed the original diagnosis. During his first hospital admission, he received 11 intravenous injections of Coley's fluid with no marked improvement, although selected nodes were greatly diminished in size following inoculation of this material. X-ray therapy was begun and 1800 r were given over the left cervical and left axillary regions and 1500 r over the anterior and posterior mediastinum.

During the ensuing period through March 1948, the patient received multiple courses of roentgen radiation for recurrences of adenopathy and other associated symptoms. He received approximately 1800 r per port at a daily dose rate of 300 r over both cervical and axillary regions, over multiple areas on the back and chest, over the left upper arm and over the epigastrium. In March 1948, 600 r were given over the anterior and posterior liver areas. Therapy over the liver was administered in doses of 100 r per day. The patient received 30.5 mgm. of nitrogen mustard in February 1947 and 24.0 mgm. in February 1948. Remissions following these multiple repetitions of therapy varied

from 2 to 8 months in duration until the final admission in February 1948.

The second course of nitrogen mustard, February 1948, was not associated with any significant improvement and was followed by the rapid development of adenopathy in many areas. Additional x-ray therapy was of no value. In April, the patient was given a pyridoxine-free diet and 1 week later one oral dose of 0.5 gm. of desoxypyridoxine and 1.0 gm. of the same material on the following day. Two hours after the second dose, the patient had a generalized tonic and clonic convulsion accompanied by cyanosis. The dose was then reduced to 0.1 gm. daily for 3 days without untoward effect and without noticeable improvement. Desoxypyridoxine was discontinued since the toxic and hypothetical therapeutic levels appeared to be closely associated.

From February to May 1948, the patient received numerous transfusions of whole blood. On March 30, 1948 he received an intravenous injection of 100 cc. of serum obtained from the blood of V. H. (case 1).

On May 5, 1948, 36 days after the administration of the V. H. (case 1) serum, the patient developed a rapidly progressing jaundice associated with an increase in fever, liver size, and the onset of tenderness in the right upper quadrant. The jaundice became more intense 2 days after its onset, the patient became comatose and the presence of a bronchopneumonia was suspected. Two days later on May 9 the patient expired.

Postmortem examination of case 3 (E.M.F.): The color of skin of the entire body was a deep brownish yellow; the sclerae were markedly icteric. The left arm and shoulder were markedly swollen and the skin over them was taut and glistening. The abdominal cavity contained 300 cc. of amber-colored fluid. The left pleural cavity contained 1000 cc. of amber-colored fluid, the right pleural cavity 200 cc. The right lung contained several discrete grayish-yellow or red nodules scattered throughout the parenchyma. On cross section these nodules varied from 0.5 to 2 cm. in diameter. The larger ones which were characterized by a deep hemorrhagic mottling could be easily shelled out. The remainder of the lung tissue appeared pinkish-gray and dry. The left lung appeared to be compressed and contained more nodules than the right. The left lower lobe contained an area of consolidation extending from the hilus towards the periphery.

The spleen weighed 350 gm. and was firm and red in color. The cross section of the organ was reddish-pink and mottled with numerous deeply hemorrhagic round nodules measuring 2 cm. or less in diameter. The liver weighed 2170 gm. and its surface contained several raised nodules measuring 0.1 cm. to 2.5 cm. in diameter. On cross section the liver parenchyma was studded with small and large nodules. Some of the smaller ones appeared white and homogeneous. The others and most of the larger ones appeared either a mottled white and red or a homogeneous deep pink. The peripancreatic lymph nodes were markedly enlarged and deeply hemorrhagic. The lymph nodes of the mesentery were all enlarged, measuring 3.5 cm. or less in size. Most of them were white and homogeneous on cross section. The mucosa of the gastro-intestinal tract and small intestine contained numerous submucosal hemorrhages but was otherwise normal. Each kidney contained one large hemorrhagic Hodgkin's nodule which was located in the cortex. The parenchyma was otherwise normal. There was generalized enlargement of the lymph nodes of the retroperitoneal and mediastinal regions. The enlargement was especially marked in the retroperitoneal region. Some of the large nodes were a deep pinkish color, could be shelled out easily from the surrounding tissue, and were soft in consistency. The smaller nodes were firm and white.

Anatomical diagnosis: 1) Hodgkin's disease with widespread lymph node involvement and involvement of the lung, spleen, liver, and kidney; 2) bilateral hydrothorax; and 3) jaundice.

Histopathologic examination revealed Hodgkin's disease of a peculiar type in the lung, spleen, liver, lymph nodes, and bone marrow. The lesion in the lymph nodes contained large masses of atypical cells surrounded by areas of hyalinized fibrosis (Figs. 3 to 4). The cells were predominantly of the multinucleated giant cell type. The nuclei were extremely irregular and possessed numerous and giant-sized nucleoli. Numerous atypical mytoses could be noted. The protoplasm, stained with hematoxylin and eosin, was either a homogeneous pink or filled with granules appearing pale on a pink background (Fig. 4). In some cells smooth pink areas and granular masses were both present. Other cells exhibited phagocytosis of chromatin fragments. Evidence of necrobiosis was present in many cells, with pyknosis and karyorrhexis. The central areas of some

FIGS. 1 TO 2.—Sections of lymph node biopsy 4 years before death. Typical Hodgkin's granuloma containing reticulum cells, fibroblasts, lymphocytes, and scattered Hodgkin's (Reed-Sternberg) cells. Hematoxylin and eosin. Slightly reduced from × 120.

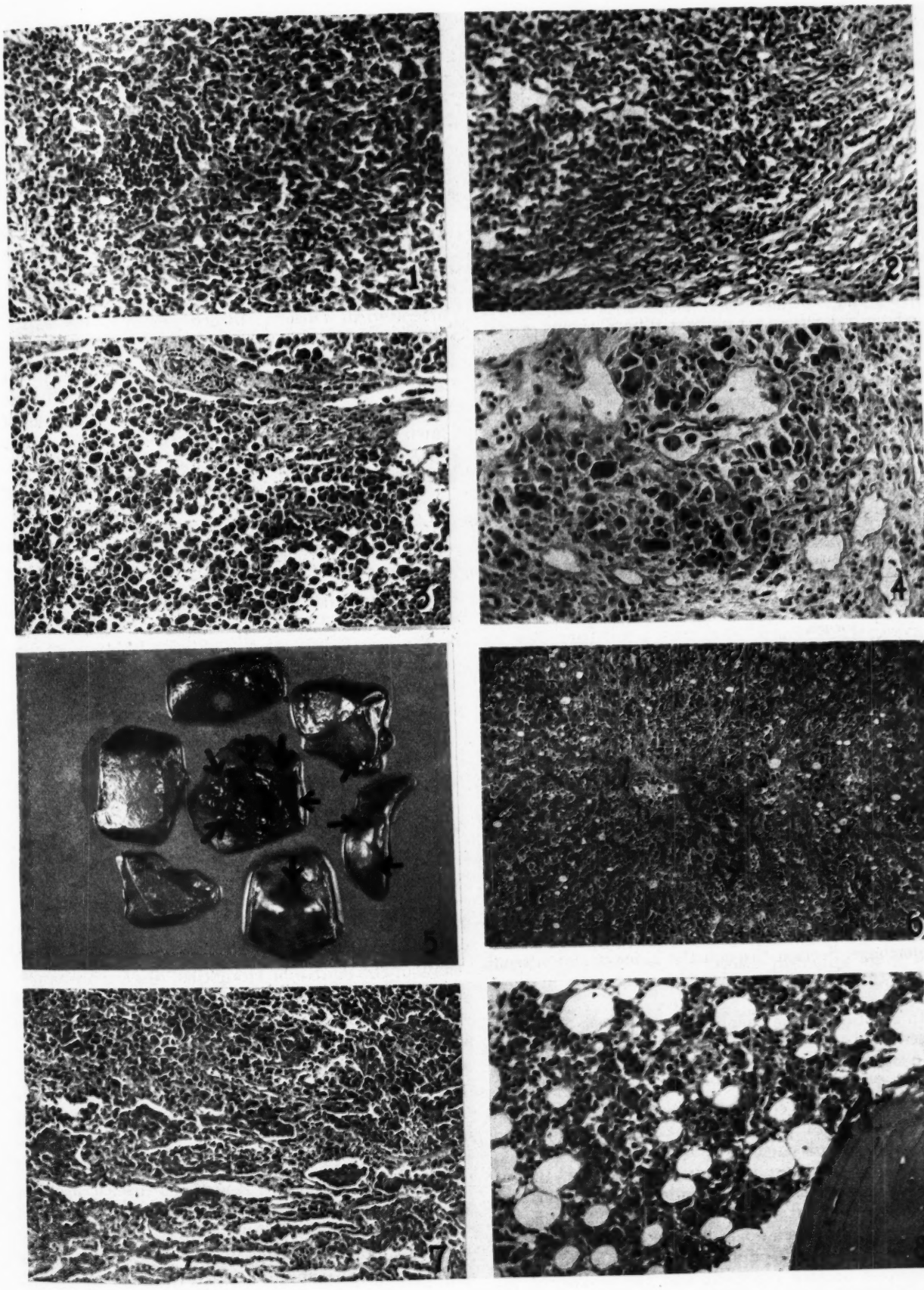
FIGS. 3 TO 4.—Sections of lymph node at autopsy. Large atypical cells containing irregular giant-sized nuclei, atypical mitoses and vacuolated cytoplasm and surrounded by hyalinized fibrotic areas. Hematoxylin and eosin. Slightly reduced from × 120 and 250.

FIG. 5.—Portions of liver at autopsy. Center, lower right, and lower center sections contain hemorrhagic Hodgkin's lesions. Upper center and upper right sections contain Hodgkin's lesions without gross evidence of hemorrhage.

FIG. 6.—Section of liver at autopsy. Leucocytic infiltration, focal areas of liver cell necrosis with hemorrhage, and scattered atypical Hodgkin's cells. Hematoxylin and eosin. Slightly reduced from × 120.

FIG. 7.—Section of lung at autopsy. Necrotic and hemorrhagic foci of Hodgkin's granuloma in areas surrounded by fibrinous exudate. Transitions between alveolar endothelial cells and atypical Hodgkin's giant cells are observed. Hematoxylin and eosin. Slightly reduced from × 120.

FIG. 8.—Section of vertebral bone marrow at autopsy. Marked erythrophagocytosis and hemosiderosis with scattered infiltration of the marrow by atypical giant-sized Hodgkin's (Reed-Sternberg) cells. Hematoxylin and eosin. Slightly reduced from × 250.



FIGS. 1–8

of the cell masses contained fields of recent necrosis with hemorrhage and leukocytes. Many of the leukocytes were of the "necrotic" type. The fibrous tissue surrounding the larger foci contained only a few fibroblasts and much collagenous material. The vascular lymphatic spaces passing from these foci contained numerous similar atypical giant cells, singly or in small groups. The foci in the liver were characterized by extensive necrosis and hermorrhage and the large atypical cells appeared singly or in small groups in the necrotic areas (Fig. 6). These cells were also found scattered throughout the sinusoids of the liver tissue. A similar picture prevailed in the lung. Here the alveoli surrounding the necrotic and hemorrhagic foci of Hodgkin's disease were infiltrated by fibrinous exudate (Fig. 7). In these exudates the proliferation of tumor cells could be observed. The endothelial cells of the alveoli surrounding the Hodgkin's foci were swollen and often could not be distinguished from the cells typical of this disease. Sections through the bone marrow contained malignant giant cells diffusely scattered, singly or in small groups, throughout the marrow (Fig. 8). Erythrophagocytosis and hemosiderosis were prominent. Other elements usually observed in bone marrow sections were present. The bone trabeculae did not appear altered. Section through the spleen revealed extensive foci of the malignant process with numerous giant cells of the type described above, necrosis and hemorrhage. Section through the kidneys and adrenal glands revealed single groups of giant cells in these tissues.

In addition to the changes described, the liver contained focal areas of necrosis which were located around the central part of the liver lobes (Fig. 6). The sinusoids appeared congested and contained, in addition to the atypical Hodgkin's cells, numerous red blood cells and white blood cells. The liver cells appeared compressed and their protoplasm stained deeply acid. Nuclei could not be demonstrated in many of the cells. Recent coagulation necrosis was surrounded by liver cells containing bile pigment and fat droplets. In these areas the infiltration of the sinusoids by leukocytes was rather prominent. In some sections of the liver these patterns of necrosis became confluent and the leukocytic infiltration appeared more diffuse. The bile ducts and periportal fields appeared normal.

Cases 4 to 24:—Among the additional twenty-one patients, who have received a total of 37 inoculations of serum and tissue extract containing the agent of viral hepatitis, thirteen have presented clinical and/or laboratory evidence of the presence of viral hepatitis. Thirty "infectious hepatitis" and seven "serum hepatitis" serum samples were used as sources of inocula.

Early observations suggest amelioration of Hodgkin's disease in seven patients of thirteen following the development of viral hepatitis. Categories of improvement in these seven cases are illustrated in Table 1. Improvement in this case is defined arbitrarily as any unequivocal favorable change in or disappearance of a symptom, sign, or laboratory finding referable to Hodgkin's disease for at least one month. The remaining six cases of thirteen who developed hepatitis did not experience any clear cut improvement in Hodgkin's disease. Eight patients who did not develop clinical or laboratory evidence of viral hepatitis following inoculation did not improve during the period of study.

TABLE 1

CATEGORIES OF IMPROVEMENT IN HODGKIN'S DISEASE FOLLOWING THE ONSET OF VIRAL HEPATITIS (CASES 1 AND 2 REFERRED TO ABOVE ARE NOT INCLUDED)

Improvement in:	7 (D.C.)	22 (D.T.)	21 (O.S.)	10 (E.H.)	14 (R.K.)	17 (R.O.)	18 (F.P.)
Adenopathy and size of tumor mass	+		+	+		+	
Pain	+		+		+		
Splenomegaly				+			
Edema of the legs			+			+	
Effusion, pleural						+	
Bone lesion, sclerosing			+				
Sedimentation rate	+	+					+
Monocyte/lymphocyte ratio		+	+	+	+		+
Lymphopenia		+	+	+	+		+
Monocytosis					+		+

DISCUSSION

The possible means by which favorable alterations in the course of Hodgkins' disease are implemented in certain cases during the simultaneous presence of viral hepatitis and Hodgkin's disease are a matter of speculation since no definitive evidence is available concerning the etiology of Hodgkin's and allied diseases. Among hypotheses which may account for the phenomena described are: (1) interference by the hepatitis agent with an enzyme system or metabolic pathway necessary to the continuance of the Hodgkin's disease process, (2) competition of the virus of hepatitis with the "tumor virus" of Hodgkin's disease, presumably unfavorable to the latter during the presence of its competitor in the body, (3) the presence in the circulating body fluids of anti-hepatitis virus antibodies or other substances which protect against

the agent or abnormal metabolic mechanism responsible for Hodgkin's disease, (4) competition between the agent of viral hepatitis and the remaining components of the Hodgkin's cell for a specific nutritional factor, or (5) the alleviating effect of viral hepatitis on certain mechanisms in Hodgkin's disease involving disturbances due to atopic phenomena.

Since no beneficial response has been noted following "obstructive" jaundice associated with Hodgkin's disease, it may be suggested that the presence of the infectious agent of viral hepatitis is a necessary component in this case. Information concerning the duration of viral hepatitis in relation to the degree of amelioration of Hodgkin's disease remains to be obtained. Clinical hepatitis was present in case 1 (V. H.) for many months. On the other hand, symptoms of hepatitis persisted for only one week in case 2 (M. C.). In the remaining seven cases, clinical and laboratory evidence of jaundice were present for varying periods of time.

Gross postmortem study in E. M. F. (case 3) indicated that, although the mechanism and etiology of the jaundice could not be ascertained, bile duct obstruction by extrinsic compression could be ruled out. Microscopic study revealed a necrotizing process involving liver cells especially of the central lobe areas. The findings (Fig. 6) were histologically compatible with those of an acute infectious viral hepatitis of "the less fulminating type" (12). The massive hemorrhages (1 mm. to 4 cm.) in association with approximately one-third of the macroscopic Hodgkin's lesions observed at postmortem in the organs and tissues of case 3 (Fig. 5) have not been observed previously by the authors in association with Hodgkin's disease, have not, to the authors' knowledge, been described in the literature in association with the concurrence of the two diseases, and may or may not represent a pathologic syndrome related to the mechanism responsible for the favorable alterations in the course of Hodgkin's disease in the small number of cases described. Although tumor hemorrhage in the presence of bacterial and viral toxins is a well known and accepted phenomenon (6) (16), insufficient evidence is available to justify a conclusion concerning the mechanism of the phenomenon described in case 3. The remissions described in cases 1, 2, 7, 10, 14, 17, 18, 21, and 22 may have been due to a subacute or chronic parasitization of certain Hodgkin's-involved segments of the reticulo-endothelial system by the agent of viral hepatitis rather than due to the acute "tumor destructive phenomenon" following an acute and fulminating episode of erysipelas (2).

The microscopic appearance of the lesions observed in case 3 confirms the gross impression of extensive necrosis and hemorrhage. An enormous proliferation or enlargement and unmasking of atypical cells resembling in many respects the giant cells (Sternberg-Reed) of Hodgkin's disease was the predominant finding. The biopsy specimen, which was obtained five years previously at another institution, revealed the average picture of Hodgkin's granuloma (Figs. 1 and 2). The foci observed in the biopsy were predominantly composed of fibroblasts, reticulum-cells, and white blood cells; atypical giant cells were uncommon. At the time of death (Figs. 3 and 4) the only cell which appeared to survive and proliferate was the large atypical endothelial cell. The bizarre appearance of the nuclei and the presence of numerous mitotic figures suggests that rapid proliferation of these cells was in progress at the time of death. Large atypical cells which appear to be similar in certain respects to these, have been referred to briefly in the literature and are said to occur following nitrogen mustard (17) and certain other types of therapy in Hodgkin's disease. The last dose of nitrogen mustard was given to E. M. F. (case 3) 12 weeks before death.

Results to date suggest that a clinical appraisal of six months or a year may be necessary to evaluate the presence of favorable alterations in Hodgkin's disease as a result of the superimposition of viral hepatitis. Death due to viral hepatitis may supervene during this same period, or recrudescence of Hodgkin's disease activity may occur and require additional anti-Hodgkin's therapy. Appraisal in some cases is further complicated during the first six months or a year after the development of viral hepatitis by an inability to distinguish clinically between fever, adenopathy, and certain other symptoms and laboratory findings due to viral hepatitis and similar manifestations due to Hodgkin's disease. Preliminary observation suggests that the favorable influence of a concomitant viral hepatitis is most pronounced during the early stages of Hodgkin's disease.

The potential dangers involved in the artificial induction of viral hepatitis in any individual or group, and particularly in individuals who are diseased, debilitated, or aged, cannot be overemphasized. For this reason it is recommended that this procedure be limited to carefully selected patients who are fully aware of the risk inherent in this unproven method of attack and that those who engage in carrying out this procedure do so with a thorough knowledge of all phases of the literature of viral hepatitis. The induction of viral hepatitis at the present time cannot be recommended as a therapeutic measure and should not be used as a

substitute for x-ray or nitrogen mustard therapy. The choice of SH rather than IH as a source of inoculum carries with it a more serious risk.

SUMMARY

Alterations in the clinical course of three patients with Hodgkin's disease presumably due to the concomitant presence of viral hepatitis are described. A preliminary description of an attempt to influence the course of Hodgkin's disease in 21 additional patients by the experimental induction of viral hepatitis is included. A number of hypotheses are advanced, one of which may account for the phenomena observed and may serve as a basis for further investigation.

ACKNOWLEDGMENT

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Morphological and Chemical Investigation of Dermal Elastic, and Collagenic Tissue During Epidermal Carcinogenesis*

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Before cancer develops in mouse epidermis influenced by methylcholanthrene, generations of epidermal cells live for months in a strangely altered kind of existence. Their lives are in a sense askew for they carry on despite many marked deficiencies and some excesses in essential materials. The features of their manner of life have been recently summarized (1).

Investigation of these epidermal cells is now being supplemented by study of accompanying changes in the underlying dermis. Thus, a new method for staining nerve endings and fibers has been devised (2, 3) and will be used. Sebaceous glands and hair follicles (4, 5), lodged in the dermis, undergo interesting modifications. The lipase activity of sebaceous glands and of dermal fatty tissues, as revealed by the Gomori reaction, is lost (6). Alterations observed in the number and fluorescence of mast cells (7) suggested operation of the spreading factor (hyaluronidase) in the breaking down of resistance to invading cancer cells possibly offered by hyaluronic acid in the dermal tissue fluid. Preliminary experiments involving injections of hyaluronidase in the vicinity of cancer transplants seem to indicate that metastasis is thereby promoted (8). On the theory that dermal mast cells may play some part in the production of the hyaluronic acid other experiments are being made (9). Thus, data are being gathered on dermal modifications with a view to building up a comprehensive picture of what happens.

In this paper morphological and chemical modifications in dermal, elastic, and collagenic tissue are reported in the hope that they will supply clues to the changing physical and chemical properties of the dermal ground substance. The literature on this subject is scanty. It has been reported (10) that human skin with carcinoma is divisible into three zones. The first is the zone of tumor growth devoid of elastic tissue; the second is the zone of cellular infiltration with decreased elastic tissue; while the third is the zone of pressure characterized by increased elastic tissue. Turning to the skins of mice, Ulesco-Stroganowa (11) induced cancer by applications of coal tar and found in the region of epithelial hyperplasia a marked increase in elastic tissue and in mast cells while about the cancer elastic tissue was absent. It is to be noted first that in both these tissues of humans and mice the investigators reported decrease in elastic tissue next to the cancer and a more distant increase, second that the conditions described were those after cancer development. There is apparently no account in the literature of the sequence of alterations in dermal elastic and collagenic tissue during epidermal carcinogenesis except one which was reported by Bierich (12) who made a study of mouse skin at 5 minute, 6 hour, 24 hour, and 5 day intervals after x-ray irradiation. But some useful information is available concerning elastic and collagenic tissue on nonmalignant conditions of the skin (13 to 24). I am grateful to Dr. E. V. Cowdry for suggestions.

MATERIAL AND METHODS

Histological data.—Female Swiss mice 4 weeks old were employed: (1) Five served as normal controls. (2) Others received applications of 0.6 per cent methylcholanthrene in benzene delivered to their backs by a No. 4 brush three times a week in exactly the same manner that is regularly done in work by others in this group. Of these 30 mice, 5 were killed after 10, 20, 30, 40, 50, and 60 days. (3) Still others received similar applications of pure benzene. Of these 10 mice, 5 were killed at 10 and 20 days.

Excised samples of skin were placed on thick paper and fixed in fresh Helly's fluid. Some tissues were embedded in tissuemat and sections, cut 25 μ thick, were stained in alcoholic orcein or in Weigert's resorcin fuchsin. Others were embedded in hard paraffin and sections, cut 8 μ thick, were colored by Mallory's stain, the quad stain, or by Foot's method. The direction of

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sections was transverse to the long axis of the body in some instances and longitudinal in others.

Attempts were made quantitatively to measure alterations observed in the elastic tissue (25). A disc, ruled in large and small squares, inserted in the ocular was calibrated by reference to a micrometer slide. At the magnification employed one of the small squares covers 25 square microns of tissue. Twenty-five of these small squares were included in each square.

Chemical data.—Female Swiss mice of the same age (4 weeks) were used and the methylcholanthrene was applied in the same manner. For elastin, chemical analyses were made of 90 mice killed in lots of 18 on 0, 10, 20, 30, and 60 days; for collagen, analyses were made of 108 mice killed in lots of 18 on 0, 10, 20, 30, 40, and 50 days from the initiation of application. Six determinations were made at each stage and the averages are given in the tables.

For normal mice, the skin was removed from almost the entire back, while from the treated mice it was only taken from the area painted with carcinogen. Panniculus carnosus was carefully removed from both with a scalpel. Epidermis was separated from dermis by the heat method of Baumberger, Suntzeff, and Cowdry (26).

The elastin content of dermis for normal mice and mice of 10, 20, 30, and 60 days, and the collagen content of dermis for normal mice and mice of 10, 20, 30, 40, and 50 days after the first application of carcinogen were determined exactly as described by Lowry, Gilligan, and Katersky (27). Since the water content in the dermis was markedly affected by carcinogen, all dermis samples were frozen and dried for 48 hours before extraction with alkali in order that the results could be calculated on a uniform dry weight basis.

The percentages thus obtained sufficed to show the relative amounts of elastin and collagen. To compare the elastin and collagen content in various stages of carcinogenesis, an absolute measurement of elastin and collagen is necessary. Hence, further work was done by taking unit areas of dermis and weighing both before and after being dried at a temperature of 110° C. (28). Unit area of dermis was taken by means of a quadrilateral razor-blade device which covers an area of 275 square mm. (11 mm.×25 mm.). The skin sample was obtained directly from the carcass of the shaved mouse by using this instrument in as uniform a manner as possible to minimize distortion. The dry weight of the dermis of a unit area of dermis was thus determined, and by multiplying it by the percentage of either elastin or collagen of the corresponding stage, the absolute weight of elastin or collagen of that special stage was obtained.

To separate the dermal part from the epithelial part of the cancer is impossible; consequently, the elastin and collagen content of the cancer were compared with that of fresh normal skin, that is, of dermis plus epidermis and the results were calculated on a wet weight basis.

OBSERVATIONS

Figure 1 illustrates the appearance of elastic tissue in a section of normal mouse skin cut parallel to the slope of the hair, that is longitudinally to the long axis of the body and likewise to the hair follicles. Part of the erector muscle is located to the right of the hair follicle in the obtuse angle formed between it and the epidermis. Elastic fibers encircle the distal portions of the hair follicles, stretch between them and along the erector muscles. Some fibers are concentrated just beneath the epidermis, especially at the attachments of these muscles, and seem, as so-called Herxheimer's fibers, to be attached to the epidermis. Individual fibers are approximately 1.2 μ in thickness.

But the actual orientation and arrangement of elastic tissue in living skin is undoubtedly somewhat different. When still living skin is freshly excised, it can be seen to shrink noticeably so that when flattened out on a piece of stiff paper for fixation it occupies a smaller area than it did originally in the animal. The extent of this shrinkage has been measured in biopsy specimens of antecubital skin by Evans, Cowdry, and Nielson (29). Skins of young persons in their thirties lost 38 to 50 per cent of their original areas. In mice this shrinkage is more difficult to determine accurately because of extreme hairiness. There is also a further shrinkage of at least 10 per cent owing to fixation and other steps in the preparation of sections. Consequently the elastic tissue of the living animal is more spread out, made up of thinner fibers, and under greater tension than Figure 1 indicates.

It is also to be noted that if the sections represented in Figures 1 to 4 were of the thickness usually employed in histopathological examinations, say 10 μ, instead of 25 μ, the amount of elastic tissue contained in them would be much less. The advantage of these thick sections is that with a binocular microscope the arrangement of fibers in depth can be better studied. Individual fibers can be traced through longer distances and alterations in their girth can be detected. The photomicrographs give only an artificial, flattened-out picture in one plane of the fibers.

By counting the elastic fibers in many 25 square micra areas a rough measure was obtained of the number of elastic fibers. The dermis selected for such determinations was located between hair follicles and at some distance from them so as to avoid the concentrations of elastic tissue in their immediate vicinity. Also, in making the counts the upper margin of the large square in the ocular as nearly as possible was superposed on the distal extremities of the layer of basal epidermal cells so that in each specimen fibers in an equal depth of dermis covered by the small squares were counted.

These counts showed a gradual and progressive

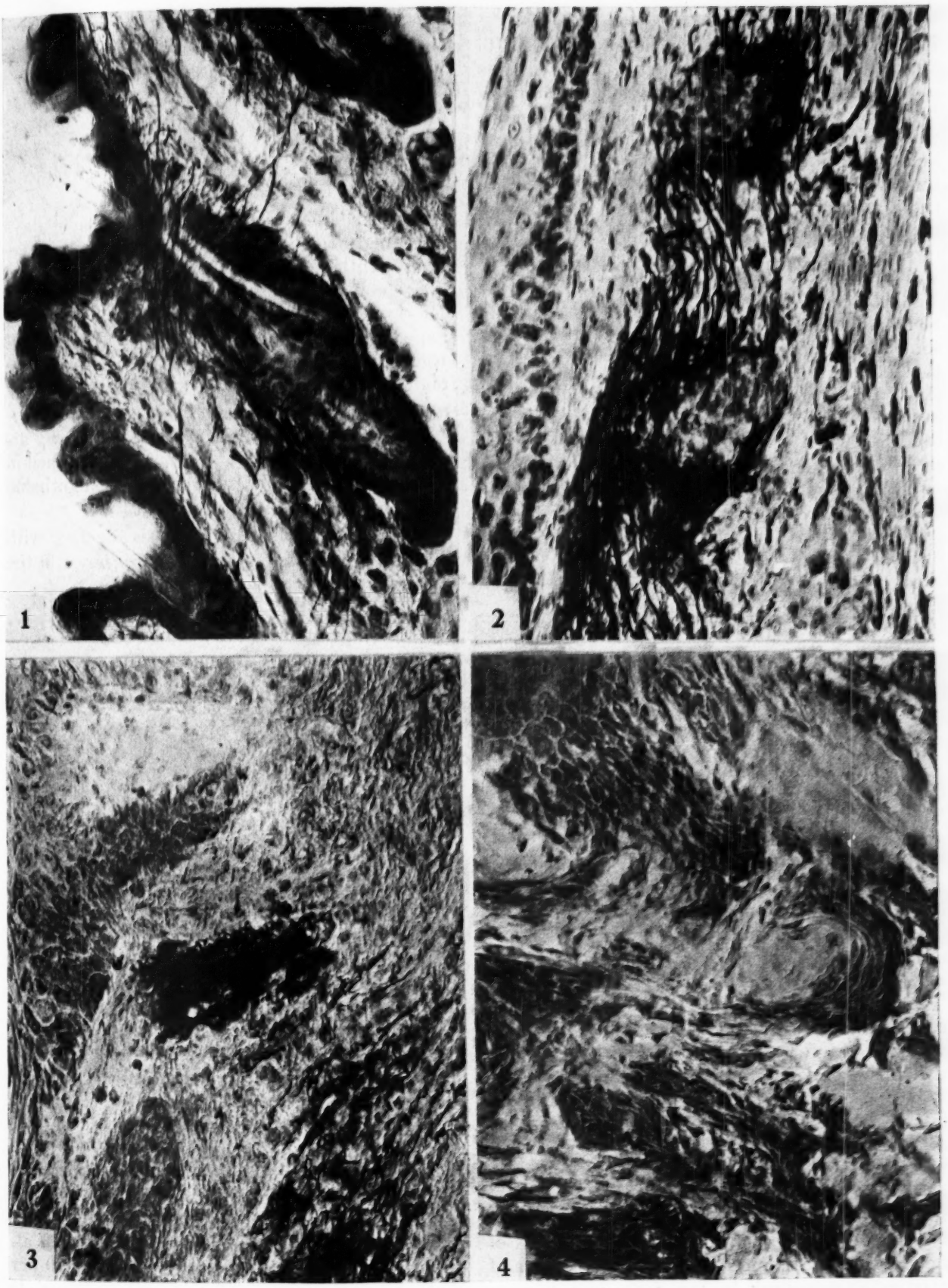


FIG. 1.—Skin of normal mouse, cut parallel to the hair current to show the elastic fibers and their relation to the hair follicles and erector pili. Mag. × 350.

FIG. 2.—Skin of mouse 40 days after initial application of carcinogen. Increase of elastic fibers cut longitudinally is shown. × 350.

FIG. 3.—Skin of mouse after 60 days. Islands of elastic tissue, (the so-called "elastica mimica"). × 350.

FIG. 4.—Skin of mouse likewise after 60 days. Masses of cancer cells with no elastic fibers. × 350.

increase of elastic fibers in specimens examined 10, 20, and 30 days after the beginning of applications of the skin of 0.6 per cent methylcholanthrene in benzene. After 10 days individual fibers became thickened to about 1.5 μ and retained this thickness to the stage shown in Figure 3. The peak in number seemed to be at 30 days for at 40 days there was a noticeable decrease which became very much greater at 50 days. The fibers became so closely bound together at 60 days that counting was impossible.

This increase in elastic fibers to a maximum at 30 days in the specimens examined is definite but some individual variations may exist. The vertical thickness of the dermis in preparations of the skins of normal untreated mice ranged from 60 to 90 μ. This thickness rapidly increased with the repeated applications of the carcinogen. In the 30 and 40 day specimens, thicknesses of 200 and 400 μ were measured. If the dermis had, on the contrary, decreased in thickness, the increase in elastic fibers might have been attributed to crowding of approximately the same number into a thinner sheet of dermis.

The appearance of elastic tissue at 40 days is indicated in Figure 3. The elastic tissue immediately beneath the epidermis is reduced but the amount stretching in a band between remnants of hair follicles is certainly increased. Some of the fibers are also of larger girth. Careful study showed a little irregularity in thickness of individual fibers. A few detached or broken ends were slightly swollen. No change could be detected in their affinity for specific stains.

At 60 days entangled masses not unlike the so-called elastica mimica in facial skin (15, 16, 17, 30) were found and are shown in Figure 2. A large mass made of deeply staining degenerated fibers is to be seen a little to the left of center. A larger and less dense mass, in which individual fibers can more clearly be distinguished, is further to the left.

In the course of epidermal carcinogenesis, the elastic fibers lose their attachments to the epidermis as this tissue becomes hyperplastic. As the hair follicles degenerate and disappear this process of detachment is advanced so that these islands of elastic tissue appear to lose form and become granular, fibrous vestiges.

That elastic tissue fades out and can hardly be found in the stroma of the resulting cancer is shown by Figure 4.

Figure 5 shows the dermis of normal mouse. It is composed of densely arranged collagenic fibers and a few connective cells, the cytoplasm of which is not easily seen and the identification of which is only marked by smaller, darker nuclei.

Figures 6 and 7 are pictures from mice 30 days after treatment with carcinogen. Though there seems a little difference between these two one thing is common, that is they become more cellular. The connective tissue cells are larger, irregular in shape, with abundant granular cytoplasm and large nuclei. The collagenic fibers appear to be fragmented in comparison with the long wavy course of that of normal dermis.

The stroma of cancer is composed of loosely arranged collagenic fibers and is much diminished in bulk in comparison with the invading epithelial cells. This is shown in Figure 8.

The results of chemical analysis together with the standard errors of the mean are shown in the following tables:

TABLE 1

PERCENTAGE OF ELASTIN AND COLLAGEN IN FROZEN AND DRIED DERMIS

	0 days	10 days	20 days	30 days	40 days	50 days	60 days
Elastin	1.75±0.22	1.57±0.06	1.88±0.14	3.06±0.16			2.23±0.11
Collagen	31.2 ±1.17	17.5 ±0.57	12.8 ±0.98	24.5 ±0.72	16.9±1.03	15.2±0.51	

TABLE 2

PERCENTAGE OF ELASTIN AND COLLAGEN IN FRESH SAMPLES

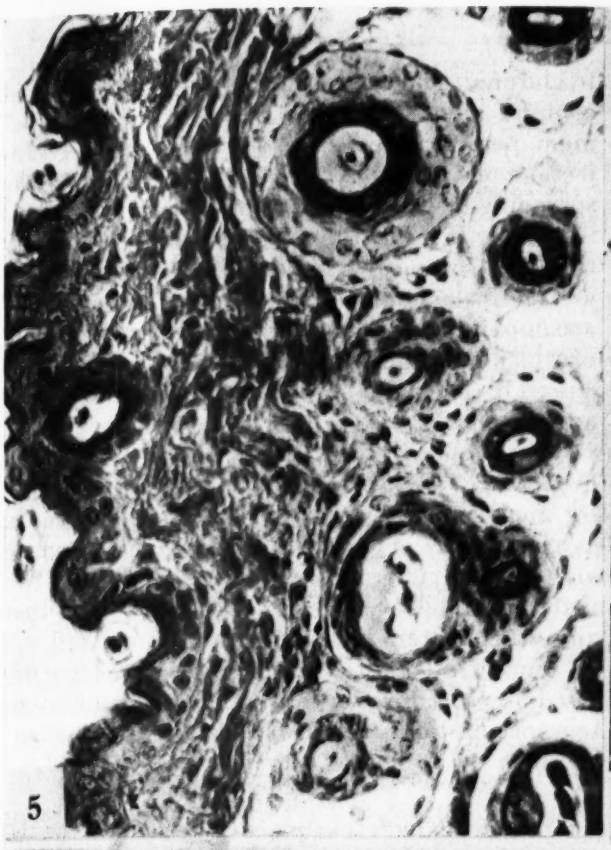
	Fresh dermis	Cancer
Elastin	1.46±0.09	0.33±0.03
Collagen	10.4 ±0.55	1.47±0.20

TABLE 3

DIFFERENCE IN WEIGHT OF A UNIT AREA (275 MM.) OF MOUSE DERMIS, BEFORE AND AFTER TREATMENT WITH METHYLCHOLANTHRENE

	Mean of wet weight of dermis in grams	Mean of dry weight of dermis in grams	Mean water content
Untreated skin	0.0496±0.0089	0.0246±0.0040	51.4%
MC treated skin	0.1712±0.032	0.0508±0.0017	71.3%

Dry weight of unit area dermis was determined on the thirtieth day. Table 3 shows a 40 per cent relative increase in water content. The weight of formed elements is doubled. Therefore in absolute weight the dermis on the thirtieth day contains almost four times as much elastin and over 60 per cent more collagen than that of normal dermis. If a curve were plotted, the elastin content would be similar though it would not quite coincide with



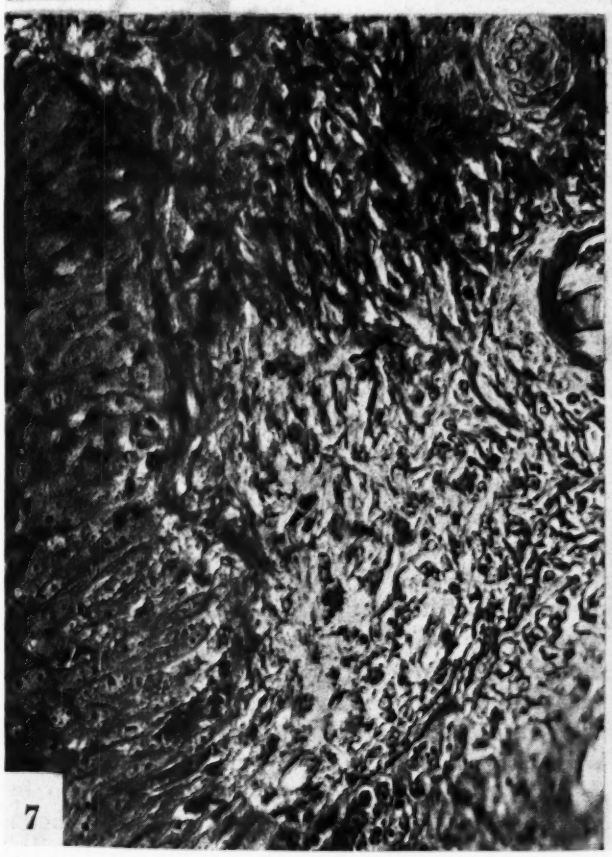


FIG. 5.—Dermis of normal, untreated mouse. There are numerous densely arranged collagenous fibers but few connective tissue cells. × 350.

FIG. 6.—Dermis of mouse 30 days after application of carcinogen. Connective tissue cells are much increased in size and in number. × 350.

FIG. 7.—Mouse dermis 30 days after application of carcinogen, to illustrate the dominant connective tissue cells.

FIG. 8.—Illustrating that the connective tissue stroma is diminished in bulk in comparison with the epithelial cells in cancer.

that obtained by plotting the fiber counts. In both there would be a rise followed by a fall. Fiber counts are impossible in collagen fibers.

DISCUSSION

Study of methylcholanthrene-induced epidermal carcinogenesis by this group has largely stressed the chemical and morphological alterations of the dermis. This report has considered quantitative shifts in some of the connective tissue elements of the dermis under the standardized conditions employed here.

An initial increase in amount of dermal elastic and collagenic fibers after exposure to methylcholanthrene has been described; this is followed by a loss of fibers. Little significance is attached to the time scale of alterations in fiber content since samples for analysis were taken at 10-day intervals. Thus, the increase in elastic fibers first noted in the 10-day sample may actually reflect a situation existing several days earlier, and the peak in elastic fiber content of the dermis described at 30 days may occur some time between 21 and 39 days. The essential point is that application of methylcholanthrene to the skin of the mouse results in a transient increase in both elastic and collagenic fibers followed by a pronounced drop in the content of these fibers.

In view of the fact that so little is known of the physiology of connective tissue fibers, it is difficult to attempt to evaluate the significance of the changes here reported. Three clear possibilities seem to exist. Variations in connective tissue fibers reflect alterations in the behavior of the ubiquitous fibroblasts, and in surface adsorptive capacities of the fibers. Third, and perhaps most significant, loss of a network of fibers from the dermis may facilitate metastatic activities of the squamous cancer cells.

Some information is available on the interrelations of fibroblasts and connective tissue fibers. Morrione (31) induced liver cirrhosis with carbon tetrachloride and *p*-dimethylaminoazobenzene in albino rats. In the course of fibrosis he noted a proliferation of fibroblasts. After stopping application of hepatotoxic substances, the fibrous tissue underwent resorption. He attributed the latter to the activity of liver cells, the function of which is in turn dependent upon diet. Recovery from cirrhosis is impaired by a high fat, low protein diet.

Hass and MacDonald (32) have demonstrated in tissue cultures that depression of pH restricts collagen fiber deposition and enhances resorption. This has been discussed by Cowdry (33).

In 1949, Pearce and Watson (34) analyzed human skin and concluded that, in fresh samples, the hyaluronic acid and chondroitin sulphuric acid content amount to 24.5 ± 5.7 and 26.2 ± 4.7 mgm. per 100 gm. of fresh tissue, respectively. Injection of hylauronidase seems to facilitate the spreading of cancer (8). Some change may therefore occur in dermal hyaluronic acid after application with carcinogen. But data are still lacking.

The thickening of dermis referred to is probably accompanied by edema, the possible relationship of which to changes in elastic fibers has been discussed by Dick (14); but it is not clarified by these experiments.

The chemical procedures of Lowry (27) for preparation of elastin and collagen paved the way for analysis of connective tissue fiber changes in carcinogenesis. The method for elastin is simple and depends upon the relative insolubility of this material in dilute alkali. It is to be expected that any other materials insoluble in dilute alkali will be concentrated with elastin. As yet we have devised no procedure for separation of the mucoprotein of elastic tissue from the alkali resistant residue.

SUMMARY

Marked changes take place in dermal elastic tissue during epidermal carcinogenesis induced by cutaneous applications of 0.6 per cent methylcholanthrene in benzene. The number of elastic fibers is increased 10 days after the first application. Through 20 day specimens this increase is progressive and attains a maximum at 30 days. After 10 days individual fibers thicken to a diameter of about 1.5 μ. Some become rather irregular in girth at about 40 days but in general they retain this diameter to approximately 60 days. From 30 days onward the number of fibers decreases. Following loss of their epithelial anchorages a few fibers exhibit slightly swollen ends and at 60 days all elastic tissue is reduced to fibrous islands of material in dermis otherwise free of elastic fibers. In primary cancers 60 days and more after the first application of methylcholanthrene the dermis is almost devoid of elastic fibers. This primary increase and later decrease in elastic fibers was supplemented by direct chemical analysis of elastin by Lowry's method which revealed a similar primary increase and later decrease.

Other dermal changes are those of collagenic fibers and connective tissue cells. As carcinogenesis is in progress, the dermis tends to become more cellular. With chemical analysis of collagen by Lowry's method, first a slight increase and then a decrease were found. Factors possibly effecting the increase and decrease of elastic and collagenic fibers were discussed but for elucidation of the real mechanism involved, further study is necessary.

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The Distribution of Radioactive Iodine in Rats With and Without Walker Tumor 256 After Injection of Radioactive Sodium Iodide*

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In the study of iodine metabolism, it is of interest to learn how administered iodine is distributed among the various organs and what factors control its distribution. Earlier work suggests that iodine metabolism may be affected by the presence of cancer. Thus there is a small amount of evidence, reviewed by Lerman (12), that the thyroid gland is affected by extra-thyroid cancers as indicated by measurement of the metabolic rate in man and by its histological examination in animals. Preliminary observations by us have also indicated that such an effect could be detected by use of radioactive iodine in animals with large cancers. To learn at what stage of tumor growth the effect of cancer on the uptake of iodine by the thyroid gland first appears and to learn if the distribution of iodine among other tissues is also affected, the work described below was performed.

METHODS

Animals.—Altogether, 149 male rats (Sprague-Dawley) were used to which Purina fox chow and tap water were given *ad libitum*. They weighed between 73 and 160 grams at the time of killing (Table 3), except the one larger rat which was used in measuring the total recovery of injected iodine (Table 2). Two experiments were performed. In the first, 52 rats were grouped, 2 days after arrival, according to size, 8 to a cage. Four rats from each cage were then implanted in the interscapular region with Walker tumor 256.[1] Fragments of the tumor, taken from a single rat, were inserted subcutaneously during ether anesthesia. Two rats from each cage served as controls and underwent sham-operation. The remaining two rats were unoperated controls.

In the second experiment, 96 rats were grouped, 3 days after arrival, so that each cage contained rats of sizes ranging from the smallest to the largest found in the entire lot. Half of the rats in each cage were then implanted with the tumor as before, and the other half were sham-operated to serve as controls for the rats in the adjacent cage with which they were paired.

Injection of iodide.—Each of the 148 rats received only a single injection. This consisted of 0.8 ml. per 100 grams of body weight of the radioactive sodium iodide solutions described below. It was given subcutaneously in the dorsal flank. After injection each rat was kept separately. In the first experiment, on 52 rats, injections were begun 7 days after tumor implantation and were carried out over a period of a week (Table 1).

In the second experiment, on 96 rats, injections were made at 5, 6, 11, 12, 15, and 16 days after tumor implantation. On the sixteenth day, the injection was divided between the dorsal flanks of each rat to avoid external leakage from the needle puncture which may occur if a large quantity of liquid is injected at a single site. All animals in this experiment were killed one-half hour after injection.

Preparation of tissues for analysis.—The rats were killed by bleeding from the heart under ether anesthesia. Organs and tissues were weighed to within 10 mg., except the thyroids and adrenals which were weighed to within 0.5 mg., and the livers and large tumors which were weighed to within 0.1 gm. Thyroids and adrenals were dried on 22 mm. diameter microscope cover glasses (adrenals were first minced) and measurements of radioactivity were made directly with these dried specimens. Because of loss of radioactivity through self-absorption, as noted under "analytical errors," each thyroid was then placed in 2 ml. of 1 per cent sodium hydroxide and a 0.2 ml. aliquot was later pipetted onto a cover glass and dried for measurement of radioactivity. (In the second experiment, the thyroids were not dried but were placed directly in 1 per cent sodium hydroxide.) All other tissues and organs were placed in 1 per cent sodium hydroxide to give a concentration

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[1] Dr. Clarence Lushbaugh, then of the Department of Pathology, the University of Chicago, kindly provided this tumor which has been maintained here in Sprague-Dawley rats.

equivalent to 20 mg. of fresh tissue per 0.2 ml. By this method, adapted from that of Keating *et al.* (7), homogeneous suspensions of tissue for pipetting were obtained. Single aliquots of 0.2 ml. of each sample were then pipetted onto cover glasses, dried, and their radioactivity measured. Aliquots of 0.1 ml. of undiluted serum were pipetted onto cover glasses and similarly dried and measured. To other aliquots of serum, 10 per cent trichloroacetic acid was added. The quantities of I^{131} in the precipitates were measured (2).

Radioactive materials.—Two lots of radioactive iodine[2] were employed for the two experiments. In the first experiment, two aqueous solutions of sodium iodide mixed with I^{131} were prepared, one containing 0.092 micrograms of added iodine and 22.8 microcuries of I^{131} per ml., and the other containing 85 micrograms of iodine and 23.5 microcuries of I^{131} per ml. In the second experiment the solution contained 90 micrograms of added iodine and 21.3 microcuries of I^{131} per ml. The microcurie values are calculated for the day injections were begun.[3] As standards for comparison with tissue samples further dilutions of these solutions were made, with the addition of a few milligrams of sodium iodide, and aliquots were measured onto cover glasses where they were each mixed with a drop of dilute silver nitrate solution and dried. Fresh standards were prepared weekly.

TABLE 1

SCHEDULE USED IN INJECTING AND KILLING RATS IN THE FIRST EXPERIMENT

DAYS BETWEEN IMPLANTATION AND INJECTION OF SODIUM IODIDE	MICROGRAMS OF IODINE GIVEN AS LABELED SODIUM IODIDE PER 100 GRAMS OF BODY WEIGHT	
	0.074	68
	Hours between injection of iodide and killing	
7	48 and 96	72
9	4	24 and 96
10	72	
11	24	½
12	½	4 and 48
14		⅓

Measurement of radioactivity.—The radioactivities of these dried aliquots were measured using Victoreen mica window G-M counters, inside 2 or 3 inch thick lead shields, mounted over the samples to give geometric efficiencies of 20 to 25 per cent. The counters were connected to scaling circuits of Higinbotham's design with scales of 1 to 64. In the first experiment, on 52 rats, all aliquots were measured until either 1000 impulses above background counts had been recorded or for a length of time at least equivalent to 5.5 minutes on the day injections were begun (*e.g.*, 11 minutes 8 days later). In the second experiment, on 96 rats, either 6400 impulses were counted or counting was continued for the equivalent of 9.2 minutes on the day injections were begun. Standards were counted within 3 hours of making a measurement on any sample aliquot. Backgrounds were counted at least as long as any sample aliquot.

Analytical errors.—Several measurements of analytical errors were obtained. The radioactivities of duplicate aliquots of the sera and thyroids from the 96 rats used in the second experiment were compared. The differences between duplicate aliquots, expressed as per cent of their average values, had, for the thyroids, a median value of +2.6 per cent. This indicates a small systematic error in delivering duplicate aliquots from a single pipette. Two-thirds of the differences lay within ±6 per cent of the median value. The differences between aliquots of sera had a median value of +0.5 per cent and two-thirds of the differences lay within ±4 per cent of the median value.

TABLE 2

RECOVERY OF 139 MICROGRAMS OF IODINE IN 1.57 ML. OF SODIUM IODIDE SOLUTION LABELED WITH 47 MICROCURIES OF I^{131} ADMINISTERED TO A RAT INTRACARDIALLY ONE-HALF HOUR BEFORE KILLING

	Micrograms of iodine per gram of fresh tissue	Total micrograms of iodine found in tissue
Blood (only the portion withdrawn)	1.2	8.8
Stomach and contents	3.4	17.
Small intestine and contents	0.7	6.2
Caecum and contents	0.2	1.0
Large intestine and contents	0.4	1.0
Parotid salivary gland	0.5	0.2
Pancreas	0.3	0.3
Liver	0.4	4.3
Spleen	0.3	0.1
Thymus	0.4	0.1
Kidneys	0.6	1.2
Urine	....	14.
Thyroid	44.	0.7
Adrenals	0.3	0.01
Testes	0.2	0.6
Lungs	1.2	1.1
Heart	0.3	0.3
Leg muscle	0.2	0.4
Ear pinna	0.6	0.2
Hide	0.7	29.
Hair*	0.4	2.1
Tail	0.7	3.9
Fluid from chest	1.8	5.6
Remainder	0.3	37.
TOTAL		135.1 or 97%

The rat weighed 218 grams. The radioactivities of two aliquots prepared from each tissue were measured. At least 6400 impulses were counted from each aliquot.

* Sample probably contaminated with urine.

No correction for self-absorption of radiation was made. Values for adrenals are particularly low because of the self-absorption incident to their being dried in relatively thick layers on the cover glasses for counting. Thus the 52 rat thyroids in the first experiment, prepared in similar fashion, measured an average of 75 per cent ± 10 per cent (*i.e.*, ± one standard deviation) as much as the same thyroids afterward treated

[2] The radioactive iodine used in this investigation was supplied by the Clinton Laboratories, Oak Ridge, and obtained under allocation from the U.S. Atomic Energy Commission.

[3] All absolute values for radioactivity are based upon information furnished by the Clinton Laboratories and not upon measurements made in this laboratory.

with 1 per cent sodium hydroxide and measured as indicated under "preparation of tissues."

A total recovery of 97 per cent of a small amount of radioactive sodium iodide was obtained from the tissues of a rat to which the iodide had been administered by intracardiac injection (Table 2).

RESULTS

The quantities of iodide injected covered the range from a small quantity, equivalent to about one-fourth of the total iodine that might have been present initially in the blood of one of the rats, to a thousand-fold larger, "pharmacological" quantity (9). Yet these different quantities of iodine were distributed among the tissues proportionately in similar fashion (Figs. 2 and 3). Consistent differences in the proportions of the different quantities in injected iodine were observed only in the thyroid, serum, and kidneys (Figs. 1 and 2). The rates of accumulation and discharge of I^{131} by the thyroid were more rapid with the small quantity of injected iodine than with the large quantity, although there is no evidence in these data that the time of maximum accumulation differed for the two quantities. The concentrations of I^{131} in the sera and kidneys decreased at slower rates in rats that received the small quantity of iodine.

In all cases the rates at which the injected iodine disappeared from tissues other than the thyroid, kidneys, and serum were similar for the various tissues up to 24 hours after injection. After that the rates of I^{131} loss from the ear pinna, liver, kidneys, adrenals, testes, heart, and possibly from brain and thymus were less than the rates of loss from the serum and other tissues (Figs. 1, 2, and 3). Since the data are more variable at the longer time intervals between injection and killing, these findings are not further detailed.

The high concentrations of injected iodine observed in the ear pinna with both large and small

TABLE 3

AVERAGE BODY AND ORGAN WEIGHTS OF THE 148 RATS USED IN THE TWO EXPERIMENTS

	1st experiment				2d experiment					
	Rats with large tumor	Rats with small tumor	Sham-operated control rats	Un-operated control rats	Tumor rats	Sham-operated control rats	Tumor rats	Sham-operated control rats	Tumor rats	Sham-operated control rats
Number of rats in each group	14	12	13	13	16	16	16	16	16	16
Days between time of tumor implantation and death					5 and 6		11 and 12		15 and 16	
Body wt. at time of tumor implantation	47.5	45.7	46.2	47.1	56.2	55.9	54.4	54.2	55.9	55.9
±standard error	±0.9	±1.0	±2.0	±0.9	±1.1	±1.0	±0.7	±1.1	±0.8	±0.8
Body wt. at death	113.3	106.5	107.0	112.2	85.0	83.8	120.7	119.1	144.0	147.7
±standard error	±3.9	±3.0	±2.9	±3.4	±1.6	±1.6	±2.2	±2.2	±2.7	±2.1
Organ wts. in % of body wt.										
Tumor	2.52	0.939			0.070		1.30		1.88	
Liver	5.322 (3)	5.221 (3)	5.065	4.973	4.944 (8)	4.936	4.992 (12)	5.132	5.101 (6)	5.002
Spleen	0.434 (5)	0.393 (5)	0.370	0.387	0.447 (5)	0.398	0.405 (10)	0.418	0.520 (5)	0.391
Thymus	0.317 (11)	0.346 (10)	0.362	0.355	0.378 (6)	0.369	0.337 (7)	0.360	0.326 (8)	0.361
Lungs	0.607 (7)	0.645 (8)	0.641	0.643	0.707 (4)	0.674	0.597 (10)	0.624	0.566 (10)	0.599
Kidneys	0.894 (11)	0.932 (8)	0.974	0.958	1.000 (11)	1.012	0.890 (10)	0.934	0.842 (10)	0.882
Heart	0.357 (9)	0.371 (10)	0.375	0.380	0.382 (8)	0.376	0.371 (10)	0.378	0.358 (9)	0.364
Testes	0.894 (7)	0.898 (7)	0.923	0.939	0.790 (12)	0.857	0.978 (8)	0.955	1.071 (5)	1.022
Adrenals	0.0207 (4)	0.0214 (5)	0.0205	0.0208	0.0227 (9)	0.0236	0.0200 (6)	0.0190	0.0180 (6)	0.0174
Thyroid	0.0079 (8)	0.0094 (4)	0.0090	0.0087	0.0102 (7)	0.0102	0.0083 (8)	0.0084	0.0075 (12)	0.0081
Stomach	0.476 (8)	0.485 (9)	0.506	0.493						
Forestomach	0.151 (9)	0.159 (8)	0.161	0.152						
% of grossly necrotic material in tumors	20	15					10		30	

The numbers in parentheses are the number of instances in which values for the tumor rats were lower than for the control rats.

quantities of iodide had not been anticipated. They exceeded the concentrations in all other tissues except the serum, thyroid, stomach, and forestomach, and at some times they exceeded even the concentrations in these tissues (Figs. 1, 2, and 3). Relatively high concentrations of injected iodine also occurred in the young tumor implants as compared with older, larger tumors (Fig. 4 and Table 3). Lowest concentrations were found in the brain (Fig. 3).

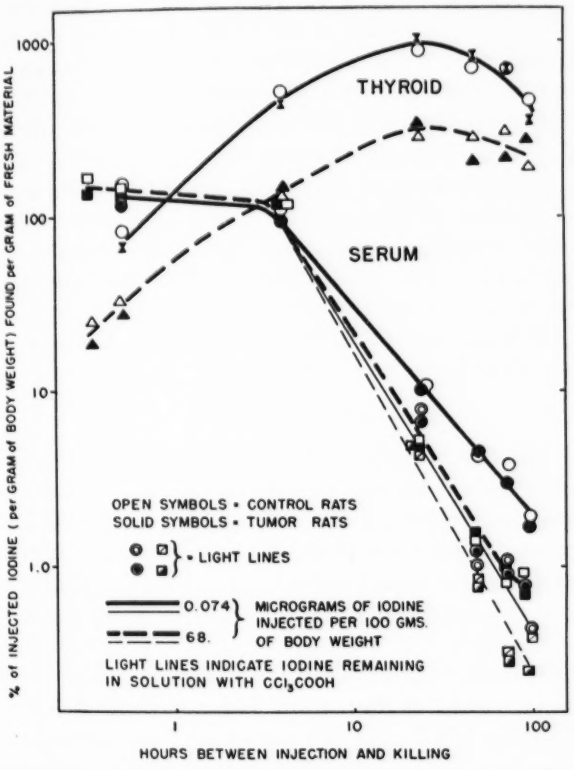

FIG. 1.—Concentrations of injected iodine found in the sera and thyroids of tumor and control rats in the first experiment. Each point represents the average of values for two rats.

During the period of 11 days in which rats were killed in the second experiment, changes were observed in the quantity of injected iodine accumulated per gram of fresh weight in half an hour by each of the seven tissues examined. The concentration of injected iodine in the thyroids increased (Fig. 4) and the concentrations in the lungs, heart, thymus, spleen, kidneys, and muscle decreased. This is made evident in Figures 5 and 6 which present the ratios of tissue I^{131} concentration to serum I^{131} concentration for the 576 tissue specimens concerned. In this 11 day period the surviving rats gained 70 per cent in body weight (Table 3).

Several differences between the tumor and control rats were observed. But no regular difference was observed between the sham-operated control rats and the unoperated controls, a matter discussed below. The tumor rats had lower concentrations of the injected iodine in their thyroids than did the control rats. In the first experiment, the thyroid of each of 9 of the 13 rats[4] with large tumors had a lower concentration of the injected iodine than the average concentration in the thyroids of the two control rats in the same group (Table 4). Lower concentrations were found in the thyroids of only 6 of the 12 rats with small tumors. The groups of rats in which the thyroids of the tumor animals had these lower concentrations were distributed evenly among those injected with the large and those injected with the small quantities of iodine. This was true of rats with small tumors and of those with large tumors, considered separately. The length of time between injection and killing was not related to this difference between tumor and control rats.

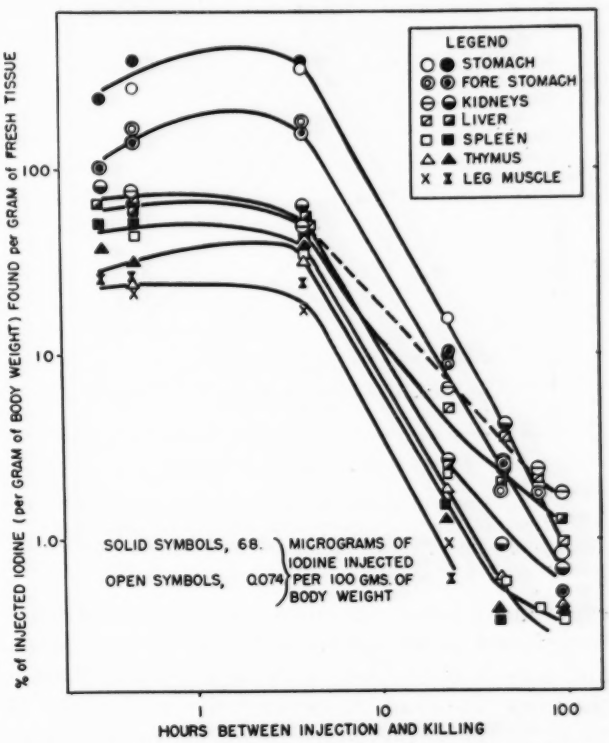

FIG. 2.—Concentrations of injected iodine found in the tissues of rats in the first experiment. Each point represents the average of values for two tumor and two control rats.

These findings suggested the second experiment. It confirmed and extended the observation that lower concentrations of injected iodine occurred in the thyroids of tumor rats than in those of paired control rats. This difference was observed in 32 of the 48 pairs of animals employed. The data, more extensive and more readily comparable than in the first experiment, are presented in Figure 4.

[4] The weight of the thyroid from one rat was lost.

The blood sera of the tumor rats also had lower concentrations of the injected iodine than did sera from the paired control rats. In the first experiment, 12 of the 14 rats with large tumors had these lower concentrations; only 5 of the 12 rats with small tumors had them. In the second experiment, the sera of the tumor rats had lower concentrations of injected iodine than the sera of the paired controls in 31 of the 48 pairs (Fig. 4). The concentrations in the sera within a day after injection were probably reflected in the tissues. Thus, in the first

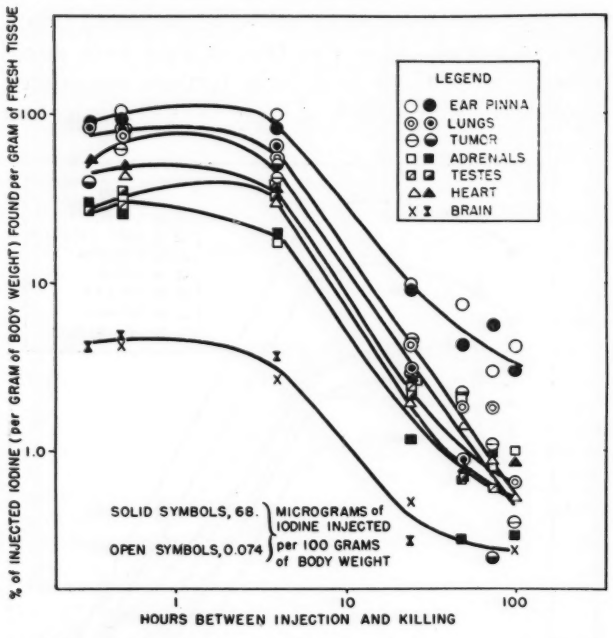

FIG. 3.—Concentrations of injected iodine found in the tissues of rats in the first experiment. Each point represents the average of values for two tumor and two control rats.

experiment, lower concentrations of injected iodine were found in the spleen, kidneys, thymus, lungs, and muscle of the rats with large tumors than in the same tissues of the control rats in 11 of the 14 pairs. In the second experiment, in the six tissues examined from 48 pairs of rats, lower concentrations of injected iodine were found in tissues from tumor rats than in those from the paired controls in the following number of instances: lungs 30, heart 29, muscle 29, spleen 29, thymus 28, and kidneys 25. But if the ratios of tissue I^{131} concentration to serum I^{131} concentration are calculated for each rat, then lower ratios are found for the tumor rats than for the controls only in the following number of pairs: lungs 20, heart 20, muscle 21, spleen 16, thymus 21, kidneys 19, and thyroid 27. The random distribution of these ratios, save for the important exceptions already noted, is illustrated in the charts (Figs. 4, 5, and 6).

Other differences between the tumor and control rats were found in the weights of some organs. A majority of the tumor rats had smaller kidneys and thymus glands and larger adrenals, spleens, and livers than did the paired control rats (Table 3). These differences were small as might be expected in groups of animals in which even the largest tumors did not exceed 3 and 4 per cent of the body weight (second and first experiments, respectively). But the differences were probably not the result of malnutrition of the tumor animals since both tumor and control rats grew at the same rate (Table 3).

DISCUSSION

The distribution of large quantities of administered iodide among the extra-thyroid tissues has been concluded by others (27) to resemble that of chloride. This concept of a distribution in the extracellular fluid has been modified to exclude excretory organs such as the kidneys, the liver, and particularly the stomach which concentrates iodide, unlike chloride, to levels in the gastric juice many times higher than plasma concentrations of iodine (9). In the present study, the similar distribution of the two smaller and widely different quantities of iodine among the majority of the fifteen tissues examined also suggests lack of selective retention at intervals of one-half, four, and twenty-four hours after injection.

The exceptions to this chloride-like distribution occurred in the present study in more instances than had been anticipated. The best known exception is the thyroid. Its ability to accumulate iodine varies tremendously with the

TABLE 4

CONCENTRATIONS OF INJECTED IODINE IN THE THYROID GLANDS OF RATS WITH LARGE TUMORS AND OF CONTROL RATS IN THE FIRST EXPERIMENT

MICROGRAMS OF IODINE INJECTED PER 100 GM. OF BODY WT.	KIND OF RAT	TIME BETWEEN INJECTION AND KILLING						
		20 min.	*30 min.*	*4 hrs.*	*1 day*	*2 days*	*3 days*	*4 days*
		Millimicrograms of injected iodine per mg. of thyroid gland						
0.074	Tumor		0.055	0.27	0.88	0.47	0.40	0.15
	Control*		0.063	0.35	0.72	0.45	0.44	0.28
68	Tumor	17.4, 16.9	20.0	89	222		149	203
	Control*	21.1	24.8	103	190	219	178	122

* Each control value is the average of values from one sham-operated and one unoperated control rat.

quantity of iodine given. Even with closely similar quantities of iodine, the concentration of radioactive iodine in the thyroids of control rats varied several-fold from one group of rats to another, whether they were given carrier-free iodine (4, 10, 18, 20, 21, 24, 25, 29) or quantities of iodine ranging from 0.1 to 500 micrograms of iodine per 100 grams of body weight (1, 8, 10, 11, 21, 22, 25, 26, 28). The stomach also concentrates iodine and, depending upon the quantity of iodine administered, may even show more of the injected iodine per gram of fresh tissue than the thyroid.[5] Except after very small quantities of iodine, a much greater proportion of the administered iodine is found in the stomach than in the thyroid as, for example, in Table 1. The two different quantities of iodine injected in the present work did not produce different ratios of stomach tissue I^{131} to serum I^{131} concentrations, as might have been anticipated from Davenport's observation that higher ratios of gastric juice iodine to plasma iodine concentrations occurred with low plasma iodine than with high plasma iodine in pouch dogs (3). Possibly the rate of blood flow was a limiting factor in the present work. That fraction of the serum I^{131} which remained in solution after the serum proteins were precipitated with trichloroacetic acid fell throughout the four-day period in a fairly constant proportion of one-half to one-third the concentration of I^{131} in the stomach tissue. But the concentration of this I^{131} which remained in solution with trichloroacetic acid failed to decrease as a simple logarithmic function of time. Such a logarithmic decrease has been noted after much larger amounts of iodine were given to man (19). In explanation two possibilities, not mutually exclusive, are suggested. Reabsorption of iodide excreted by the kidney glomeruli, the gastric mucosa, and other excretory routes may be the principal controlling factor. The rate of iodide elimination at these low concentrations may also be limited by the rate of blood flow through the tissues as has been suggested in connection with the elimination of gaseous nitrogen

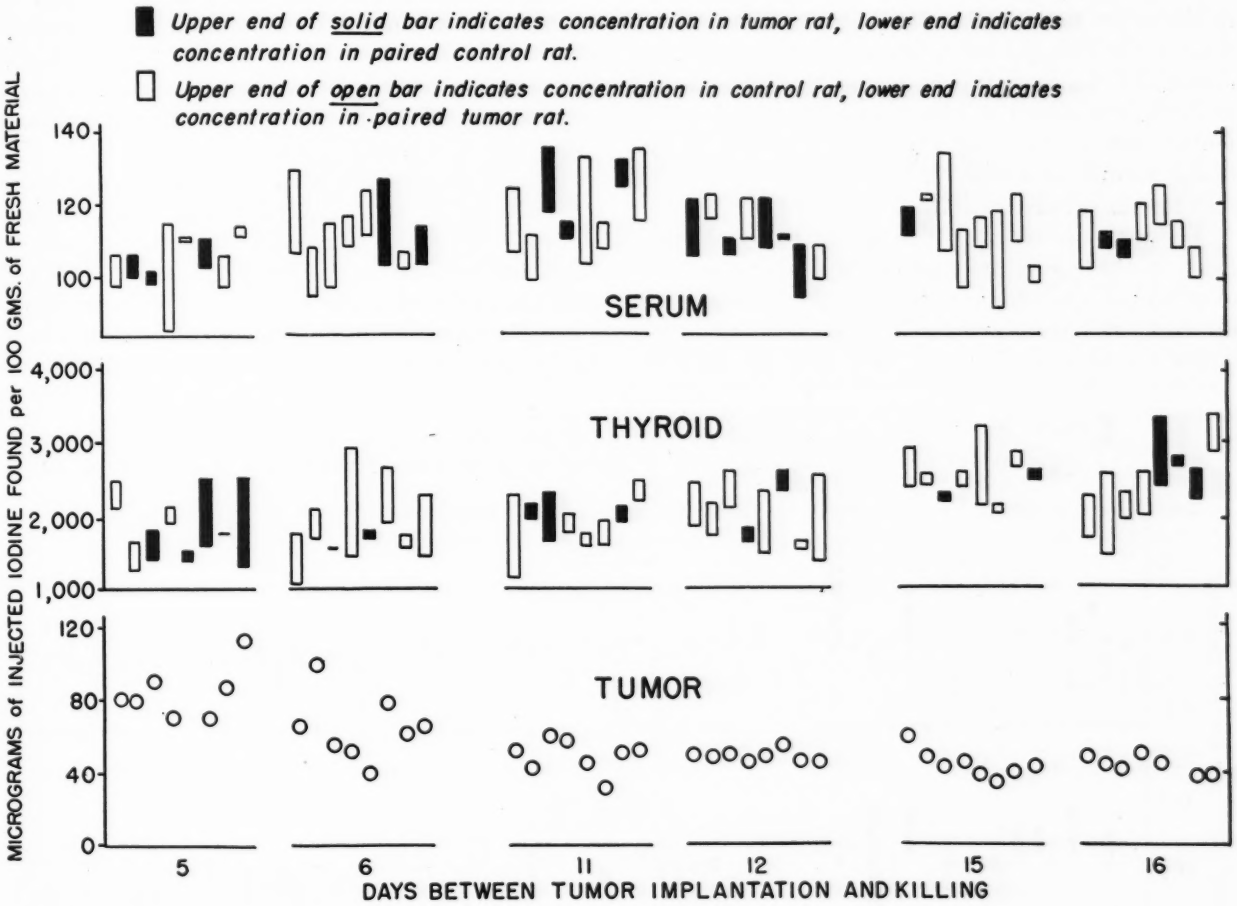

FIG. 4.—Concentrations of injected iodine found in the sera, thyroids, and tumors of rats in the second experiment. The pairs of rats are arranged for each day in order of increasing body weight from left to right.

[5] Unpublished observations.

through the lungs during oxygen breathing (6). It is of interest to note that the data from the study of the elimination of gaseous nitrogen in man, like the data on serum I^{131} in the latter part of the first experiment, gave a straight line when plotted upon full logarithmic paper but not on semi-logarithmic paper (23). It would also be of interest to learn how the proportion of injected iodine per gram of kidneys became and remained higher after injection of the small quantity of iodine than after the large quantity, without the occurrence of systematically higher concentrations of injected iodine in the other tissues as well. A large part of the iodine in the kidneys was probably in the urine they contained. In dogs it was found that the kidney medulla contained a higher concentration of injected iodine than did the kidney cortex or the blood serum, and the bladder urine contained a still higher concentration.[5]

The ratios of tissue I^{131} to serum I^{131} concentrations in the ear pinna and in the liver were both over 50 per cent higher than would be suggested by data obtained after administration of much larger quantities of iodide (27) and by data on skin and liver chloride concentrations in rats (15). The proportion of the injected dose found per gram of liver was similar to that reported to occur after oral administration of approximately 250 micrograms of iodine as potassium iodide per 100 grams of body weight (21). The I^{131} concentrations in these two tissues were elevated in the earliest samples taken, precluding the probability that they represented I^{131} already incorporated by the thyroid into thyroxine which was being excreted by the liver or accumulated in the skin (5). Whether the I^{131} accumulated in the skin or cartilage of the ear pinna is not known. Binding of chloride by cartilage has not been suggested although cartilage is known to bind sodium (14). The elevated concentration of I^{131} found in most of the young, small tumors is also unexplained.

The brain and the testes both contained lower proportions of the injected iodine than relevant data (15, 27) would have suggested. Both tissues are known to be penetrated slowly by chloride (14), but here the ratios of tissue I^{131} to serum I^{131} concentrations throughout the entire four-day period were half or less of the ratios reported for chloride (15)

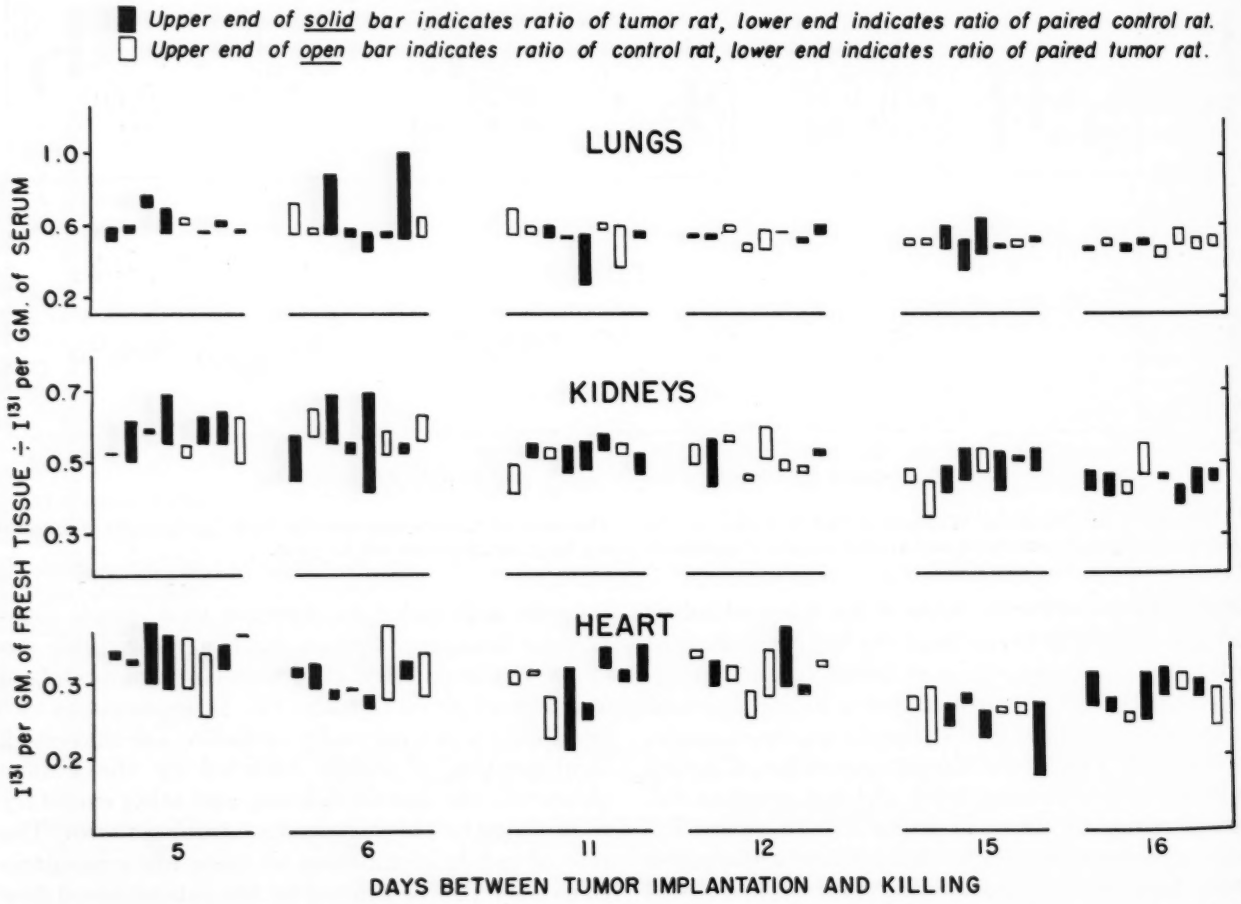

FIG. 5.—Ratios of tissue I^{131} concentration to serum I^{131} concentration for the lungs, kidneys, and hearts of the 96 rats in the second experiment. The pairs of rats are arranged for each day in order of increasing body weight from left to right.

and for large quantities of iodide (27). The ratios found here for brain closely resemble those reported for rabbit brain after subcutaneous injection of carrier-free iodine (21) and the ratios of less than 1 to 20 observed in dogs.[5] But the testes of the rabbits in the experiment cited (21) contained concentrations of injected iodine exceeding those in the serum at intervals of 1 to 8 days after injection, in contrast to the much lower proportions observed here in rat testes. Whether this difference resulted from the different quantities of iodine administered or was an age or species difference is unknown.

Decreases in the chloride concentrations in cardiac and skeletal muscle during growth have been observed and interpreted as decreases in the proportion of extracellular fluid (13). The present observations confirm and amplify this finding, for progressively decreasing ratios of tissue I^{131} to serum I^{131} concentrations occurred for six tissues during the 11 days covered by the second experiment. Only the thyroid proved exceptional, for the concentration of I^{131} in it relative to that in the serum increased, either as a result of growth or from other causes. These phenomena which accompanied growth must have added to the variability of the data obtained in the first experiment in which groups of animals of different ages were arranged at random with respect to the quantity of iodine injected and to the length of time between injection and killing.

The careful grouping of tumor and control animals in this work revealed differences in iodine distribution and in organ weights between the tumor and control animals but not between the sham-operated and unoperated control animals. Operative procedures at sites distant from the thyroid are known to produce increases in the blood iodine of man and of rabbits (16), but the procedure used here may have had effects of too brief duration or too slight for detection. A difference between tumor and control rats in iodine distribution was first observed[5] as a decreased uptake of I^{131} by the thyroids of mice and rats bearing larger and more necrotic tumors than those dealt with in the experiments described here. Evidence that tumors do or do not affect the thyroid of the host is scanty and is concerned mainly with hosts bearing large tumors (12). Our interest was in seeking effects of young, small tumors. The lower concentrations of I^{131} in the thyroids of tumor than of control rats were found in the present experiments with smaller

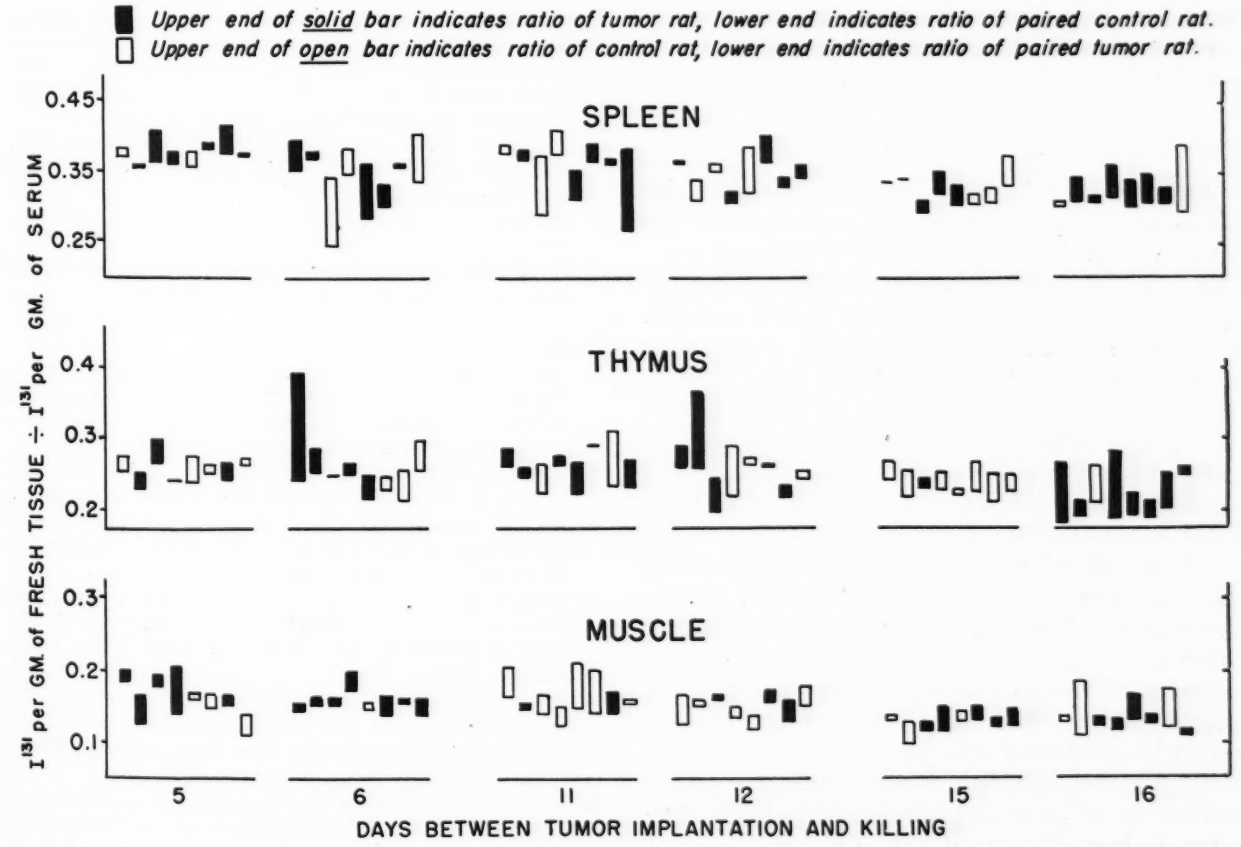

FIG. 6.—Ratios of tissue I^{131} concentration to serum I^{131} concentration for the spleens, thymuses, and muscles of the 96 rats in the second experiment. The pairs of rats are arranged for each day in order of increasing body weight from left to right.

tumors than we had previously employed. It also became evident that these lower concentrations occurred in other tissues of the tumor rats and in their blood sera. The origin of the lower thyroid concentrations of I^{131} in these tumor rats thus lies not so probably in thyroid dysfunction as in something common to the other tissues and the blood serum as well. This may be a slower rate of absorption of I^{131} from the site of injection, or a more rapid elimination through the kidneys, or it may be the accidental injection of less iodine into the tumor animals than into the controls, in spite of the care taken to treat them alike.

Differences in the organ weights of tumor and control rats are among the earliest indications of systemic effects of some tumors. In the present work, the observed differences were small and, taken singly for each organ, might not prove convincing as evidence of systemic effects. But considered together and with the knowledge that similar and larger differences have been observed in rats bearing larger tumors, both here and in other laboratories (17), they provide appropriate indication that the tumors were influencing various organs. Whether this influence was due to the presence of live, growing tumor cells or of necrotic tissue is not known. Some necrotic tumor material is probably present in these tumors throughout their growth, although in greater proportion in implants beginning to grow and in larger tumors.

SUMMARY

At intervals up to 4 days after subcutaneous injection of young male rats with sodium iodide labeled with radioactive iodine, the animals were killed and the concentrations of the injected iodine in a number of their tissues were measured. It was found that: 1) The majority of rats with implants of Walker tumor 256 had lower concentrations of the injected iodine in their thyroid glands and other tissues than did control rats grouped with them according to size and treated similarly. These differences probably resulted from lower concentrations of the injected iodine in the blood sera of the rats with tumors. The cause of this difference in the sera is undetermined. 2) The distribution of injected iodine was like that of chloride during the first day after injection in the majority of tissues examined. Concentrations of injected iodine were higher than would be suggested by published data on the chloride content in the thyroid, stomach, forestomach, ear pinna, and liver, and lower in the brain and testes. 3) The concentration of injected iodine in the tissues at one-half hour after injection changed during the 11 day period in which animals were killed. The concentration in the thyroid increased, and the concentrations in the other tissues decreased. The decreasing concentrations suggest decreasing proportions of extracellular fluid. In this 11 day period the surviving rats increased 70 per cent in body weight. 4) The decrease in concentration of that part of the injected iodine which remained in solution after precipitation of the serum proteins with trichloroacetic acid did not occur as a simple logarithmic function of time during the 4 days after injection. This was true after injection of either 0.07 or 70 micrograms of iodine per 100 grams of body weight.

ACKNOWLEDGMENTS

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The Effect on the Embryo of Continued Serial Tumor Transplantation in the Yolk Sac

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Heilman and Bittner (1) reported that the serial passage of a mouse mammary carcinoma cultivated in the yolk sac of embryonated eggs was associated with an increasing mortality rate of the chick embryos. They found that the number of embryos surviving through the ninth day was 63 per cent after 4 to 5 egg passages, 56 per cent after 8 to 9, 40 per cent after 12 to 13, and 28 per cent survived after 16 egg transfers. The experiment was continued through 20 egg transplant generations. The reason for this increasing lethal effect on the chick embryo was considered to be obscure.

Armstrong and Ham (2) have made an extensive study of the reactions of the chick embryo to the serial passage through the egg by yolk sac implantations of a mouse mammary carcinoma. They too reported an increased chick mortality with the increasing number of tumor passages. It was considered on the basis of the chick histology that the lethal influence was mediated through an anematizing effect which was possibly due to some agent associated with the tumor process.

The yolk sac method of cultivating tumor tissue was first reported from this laboratory in 1942 (3, 4). Since that time, egg-cultivated tumor tissue has been produced continuously for use in various research projects. One strain of mouse mammary carcinoma has been carried in embryonated eggs by serial implantations for more than 7 years. Many other tumors, both rat and mouse, have been grown continuously in eggs for periods of months and years (5).

It has been noted that there is a tendency for a heterologous transplant of tumor tissue in the yolk sac to affect the host embryo more adversely after several passages through the egg. At the same time the tumor tissue does not undergo the slightest observable morphological change. Further, the adverse effect is irregular in its manifestation in the eggs of a particular experimental group. Even after 150 continuous tumor transplant generations many of the eggs of an experiment are free from the toxic effect.

It seemed important to obtain further data on this transfer toxic effect; first, because it influences the size and variability of egg-cultivated tumors and must be considered when the tumors are used in chemotherapeutic experiments, and second, it furnishes further evidence for the tumor agent or virus concept. The present paper is concerned with evidence bearing on the problem which has been collected in the course of many hundreds of experiments with the cultivation of tumor tissue in the chick embryo yolk sac.

MATERIALS AND METHODS

dba and C3H mammary carcinomas were used in this study. Some observations were made on eggs bearing transplants of the rat tumor, Walker 256. The mouse tumors originated spontaneously in breeding stock and had been carried for numerous transplant generations in mice before they were used in eggs.

The method of yolk sac cultivation was essentially as described in the latest report (5). A few minor modifications have been adopted. For the past year C3H and dba tumor tissues have been inoculated into the eggs in dilutions of 1:8 instead of the 1:4 dilution used previously. The size of the egg tumor was not affected by using the more diluted suspension but where large numbers of eggs are used the amount of tumor required for inoculation represents an important item in the day to day expenditure of time and materials.

The C3H homogenized tumor tissue has lately been suspended in 0.85 per cent saline instead of a mixture of egg white and saline recommended before. The dba tumor which has been carried in eggs continuously for 7 years is still inoculated into the eggs suspended in egg white and 0.85 per cent saline in the ratio of 1:3.

It has been found that the egg mortality associated with tumor inoculation can be materially decreased by dipping the inoculating needle into alcohol and flaming between injections of individual eggs. The same 1 cc. syringe, with this precaution, can be used for 10 or 12 eggs. There is a tendency for particles of egg shell to stick to the needle as it is withdrawn from the egg thus possibly infecting the suspension from which the next inoculum is obtained. Also individual eggs are not infrequently carriers of infectious agents which can be spread to several eggs if the needle is not sterilized between injections.

The reaction of the chick embryo to repeated passages of tumor tissue through the yolk sac was evalu-

ated by changes in egg mortality and by the presence of gross malformations and other pathological disturbances in the chick. Data on changes in tumor size at harvesting were also obtained.

To test the effect of continued tumor cultivation in the yolk sac on chick mortality and tumor size, data were obtained from two series of experiments with egg-cultivated C3H mammary carcinomas. Eighteen experiments involving 1837 eggs were made with eggs bearing a tumor transplant serially transplanted in the yolk sac for 42 to 46 generations, and a series of 14 experiments involving 2271 eggs was made with the same tumor carried in eggs 2 to 5 transplant generations. The progressive chick mortality and the tumor size at harvesting were recorded and compared for the two series. Observations were made on the appearance of lesions and malformations in the chick embryos of eggs bearing the dba mammary carcinoma which has been carried continuously by yolk sac cultivation for more than 150 transplant generations and compared in this respect with embryos from eggs bearing the same tumor after being passed through the host mouse.

As Table 1 shows, the experiment was continued until the 14th day of total incubation or 10 days after tumor inoculation. About 5 per cent of the embryos of the 42 to 46 transplant group manifested various morphological aberrations or other evidence of pathological disturbance. Many chicks exhibited a generalized edematous-like condition. The body was distended and more transparent than normal. The chick embryos of the 2 to 5 tumor transplant group rarely showed such disturbances.

The effect of repeated tumor passages through the yolk sac on chick embryo and tumor size is is recorded in Table 2. It will be seen that tumor size is associated with a decrease in the weight of the host chick embryo (6). Hence the chicks bearing the smaller tumors should have been larger than those from the eggs carrying more tumor tissue.

TABLE 1

PER CENT MORTALITY OF TUMOR-BEARING EGGS ON SUCCESSIVE DAYS AFTER INOCULATION

No. of experiments	No. of eggs inoculated	Days after inoculation (Mortality in per cent) 1	3	4	7	8	10
		Tumor continuously in eggs 2 to 5 transplant generations					
3	531	5.8	24.0	28.7	36.1	37.8	45.1
4	707	4.6	15.4	23.4	31.4	36.2	42.4
3	473	2.2	15.0	20.3	26.2	31.7	38.0
4	560	1.3	12.4	15.7	22.8	26.9	33.4
Total 14	2271	Average 3.5	16.7	22.0	29.1	33.2	39.7
		Tumor continuously in eggs 42 to 46 transplant generations					
4	399	9.4	25.5	30.6	54.3	56.8	64.1
5	574	10.4	30.0	35.3	54.9	56.7	63.3
4	350	6.3	42.3	44.4	57.6	58.7	60.5
5	514	6.9	28.7	34.6	52.3	54.1	57.0
Total 18	1837	Average 8.3	31.6	36.2	54.8	56.6	61.3

RESULTS

C3H mammary carcinoma.—Table 1 and Figure 1 summarize the data obtained with respect to the effect of repeated tumor passage through the yolk sac on the mortality rate of the chick embryo. It is shown that more deaths occurred in embryos of eggs bearing tumors serially transplanted 42 to 46 generations than were recorded for the embryos of eggs containing tumors in the 2 to 5 transplant generation. On the first day after the eggs were inoculated with tumor tissue, the average death rate in the 42 to 46 transplant generation group was 240 per cent of the 2 to 5 transplant group. For the 3d, 4th, 7th, 8th, and 10th days after tumor inoculation the corresponding percentages were 187 per cent, 164 per cent, 188 per cent, 170 per cent, and 157 per cent.

size was reduced in the 42 to 46 tumor transplant group to less than one-third of the weight as compared to tumors of the same age from eggs bearing the 2 to 5 tumor transplant generation. The weight of the chick appears not to have been affected. However, as shown previously, increasing tumor

TABLE 2

COMPARISON OF TUMOR AND CHICK WEIGHTS AS AFFECTED BY THE NUMBER OF TUMOR PASSAGES THROUGH THE YOLK SAC, MEASURED 10 DAYS AFTER TUMOR INOCULATION

No. of transplant-generations in yolk sac	No. of experiments	No. of tumors and chicks weighed	Average weight of tumors (gms.)	Average weight of chicks (gms.)
42–46	18	223	0.47	6.45
2–5	14	289	1.34	6.49

dba mammary carcinoma.—Observations have

been made on the reaction of the chick embryo to the dba mammary carcinoma now in its 8th year of continuous yolk sac transplanation or more than 150 transplant generations. It has been found that most of the embryos of the eggs surviving to the 15th to 17th days of incubation were abnormal and exhibited various pathological effects. Figures 2 and 3 illustrate some of the typical lesions which affected chicks growing with these tumors. The blister-like condition shown in the illustration is very common but various other abnormalities are frequent.

In the 7 years that the dba mammary carcinoma has been cultivated serially in eggs there has been

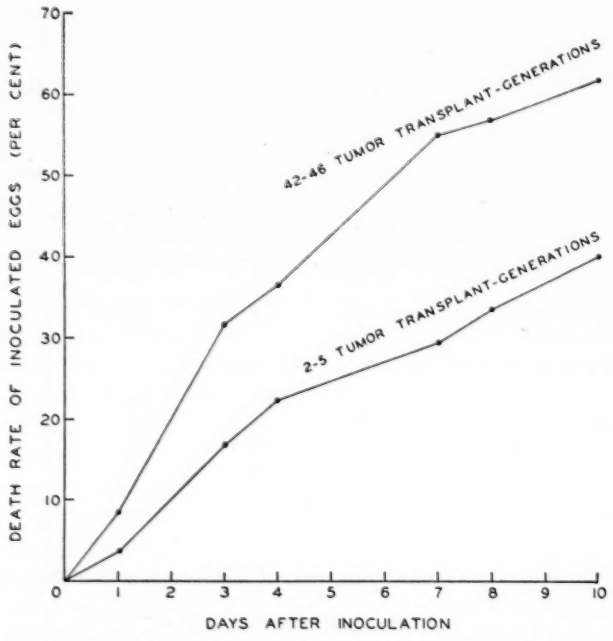

FIG. 1

a gradual increase in the number and severity of the abnormalities affecting the host chick embryos. About 70 per cent of the embryos surviving to the 14th day of total incubation, or 10 days after tumor inoculation, have been adversely affected. Yet many chicks manifesting severe toxic effects have survived to the 17th or 18th day of incubation. The type of lesion in the embryo associated with the growth of this tumor was not found in egg cultures of the C3H mouse mammary carcinoma or the rat tumor, Walker 256.

With the dba mammary carcinoma, as with the C3H and the rat tumors, the toxic effect on the chick embryo has been irregular from egg to egg of a particular inoculation group. In each experiment 10 to 20 per cent of the eggs have contained vigorous chicks and tumors. The tumor size has been much below average in the eggs showing the transfer toxic effect.

There has been no observable change in the morphology of the dba tumor during the years it has been carried by egg cultivation. It has continued to grow rapidly in the host mouse. Further, one or two transplant generations in the mouse have removed the toxic effect on the embryo from subsequent cultivation in eggs for the next 5 or 6 transplant generations.

The rat tumor was carried for 80 transplant generations or about 3 years continuously by yolk sac inoculation. The effect of repeated passage through the yolk sac on the chick embryo was similar to that recorded for the mouse tumors.

DISCUSSION

This report in conjunction with that of Heilman and Bittner (1) and Armstrong and Ham (2) would seem to establish definitely the fact that repeated passages of rat and mouse tumor tissue through the yolk sac adversely affects the host chick embryo. For the first few transplants the yolk sac cultivated tumors influence the chick embryo in about the same manner as a growing tumor transplant affects the natural host. The blood hemoglobin concentration becomes reduced as the tumor increases in size, as it does in a mouse or a rat bearing a tumor (7, 8), and in addition the growth of the tumor inhibits the growth rate of the embryo (6). But the embryo may develop vigorously in association with a tumor weighing 2 or 3 grams and often will hatch if allowed to do so. When these rat and mouse tumors are continuously cultivated in the yolk sac a new factor enters the tumor-embryo relationship. There is a rise in the mortality of the inoculated eggs. The yolk sac tumor attains a smaller average size with a marked increase in size variability from egg to egg, and the embryos become subject to various abnormalities and lesions.

The toxic effect appears irregularly in the tumor-bearing eggs as the number of tumor yolk sac passages increases. The C3H mammary carcinoma, used in some of this work, rarely affects the embryo before the 6th passage through the egg. An occasional embryo is affected adversely as early as the 4th tumor transplant generation. This tumor cultivated in the yolk sac has been used extensively here in cancer chemotherapy studies (9) so that observations have been made on many thousands of live embryos. Usually in the chemotherapeutic studies, the tumor-bearing eggs have been opened on the 14th day of total incubation. In order to have large uniform tumors and good survival a particular egg tumor is not continued beyond the 6th transplant generation in eggs. One passage of

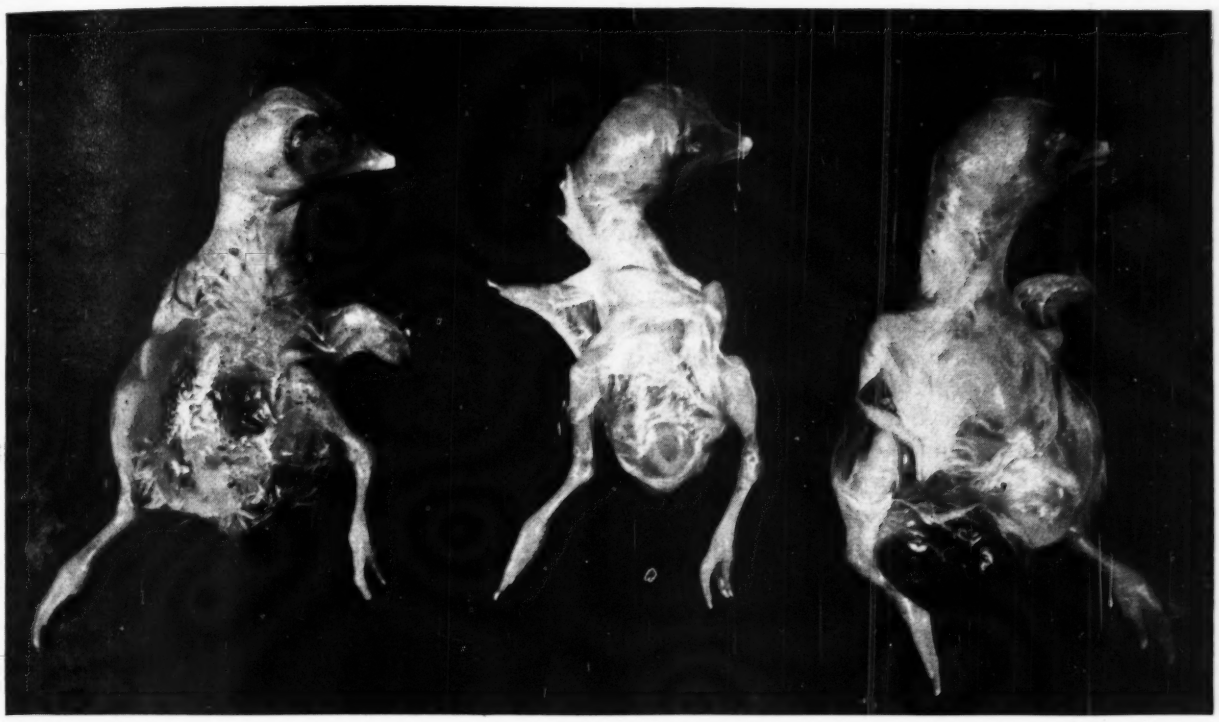

FIG. 2.—Types of lesions occurring in chick embryos as a result of serial tumor transplantation in the yolk sac. 15th day of incubation. Center chick was taken from an egg bearing a 3 gram tumor and is relatively unaffected. The two other chicks were from eggs containing tumors weighing less than one gram. (Photographed by Dr. Irving Galinsky.)

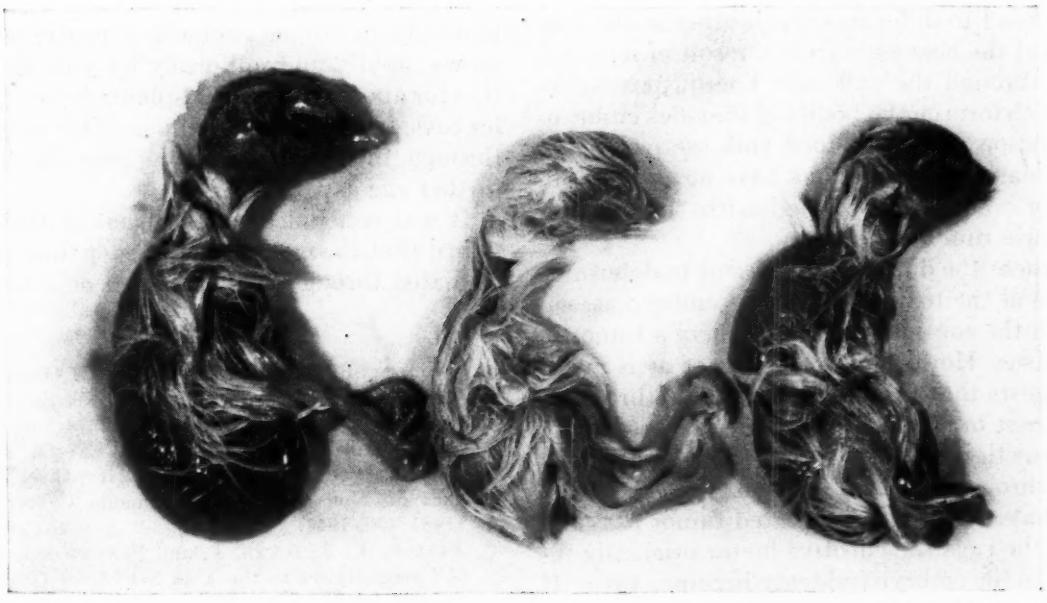

FIG. 3.—Types of lesions occurring in chick embryos as a result of serial tumor transplantation in the yolk sac. 17th day of incubation. Center chick was taken from an egg bearing a 4 gram tumor and is relatively unaffected. The two other chicks were from eggs containing tumors weighing less than one gram. (Photographed by Dr. Irving Galinsky.)

the tumor back through the normal host, the C3H mouse, destroys the transfer toxic effect. The tumor can then be reimplanted in the yolk sac for another 6 transplant generations.

It is important to note that the tumor transfer toxic effect on the embryo does not act uniformly on the eggs of a particular experiment. In a group of eggs all inoculated with the same suspension of a tumor that has been cultivated in eggs long enough for the toxic effect to appear, the affected embryos differ in the degree of the abnormalities and many are completely unaffected. The dba mammary carcinoma after being carried for more than 7 years by yolk sac cultivation differs markedly in its effect on the individual embryo. Chick embryos surviving to the 16th and 17th day of total incubation exhibit various degrees of abnormalities such as are illustrated in the figures but a few embryos of normal appearance are found in association with tumors weighing several grams.

The lack of uniformity in the transfer toxic effect plus the fact that many seriously affected embryos survive long enough for the tumor to make good growth have made it possible to continue serial transplantation in the yolk sac indefinitely. In each transplant generation there is a screening out of the inoculated eggs most affected by the tumor transfer factor.

It has been noted that the rat tumor, Walker 256, and the dba and C3H mouse mammary carcinomas tend to differ from each other in the way they affect the host embryo as a result of repeated passage through the yolk sac. The blister-like lesions which form on the bodies of the chick embryos in association with continued yolk sac passage of the dba mammary carcinoma have never been observed in embryos inoculated with the rat and C3H mouse tumor series.

At present the data are insufficient to determine the cause of the toxic effect on the embryo associated with the continuous cultivation of a tumor in the yolk sac. However, such evidence as is available suggests that the effect is mediated through a tumor agent or virus. Some particular product of tumor growth is given off into the blood and is circulated through the host embryo. It must be assumed that as a result of repeated tumor passages through the eggs the effective factor originally innocuous to the embryo suddenly becomes toxic. It is well known that viruses tend to change in their biological effects when placed in a changed environment (10).

Several papers have been published from this laboratory which were concerned with evidence for the tumor agent or virus concept of cancer causation (11 to 15). Many of the data contained in these reports were based on experiments with the same dba mammary carcinoma which furnished part of the data for this paper, and the severe pathological effects which the growth of this tumor produces in the host chick embryo as a result of numerous serial transplantations in the yolk sac provides further support for the thesis of the earlier work.

SUMMARY

The toxic effect on the chick embryo associated with the serial cultivation of tumor tissue in the yolk sac reported by others has been confirmed in this study.

Egg-cultivated rat and mouse tumors after serial passage through the yolk sac induced pathological changes in some of the chick embryos of each inoculation group. This effect was extended and became more severe with continued serial cultivation of these tumors in the yolk sac. Concomitant with the appearance of the toxic effect the yolk sac tumors were much reduced in size.

One of the mouse tumors, a dba mammary carcinoma, has been cultivated continuously in eggs for more than 7 years. Most of the embryos associated with the growth of this tumor at the present time manifest severe lesions but also in each inoculation group 10 to 20 per cent of the eggs contain vigorous chicks and tumors.

A C3H mammary carcinoma has been used extensively in tumor chemotherapeutic studies. It grows rapidly and uniformly by yolk sac cultivation for about 6 serial transplants before the transfer toxic factor begins to appear. One passage back through the host mouse prepares the tumor for further egg experiments.

It was concluded on the basis of the data obtained that the egg tumor transfer toxic effect was mediated through a tumor agent or virus.

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The Carcinogenic Activities of Certain Analogues of 2-Acetylaminofluorene in the Rat*

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2-Acetylaminofluorene, a carcinogen originally discovered by Wilson, DeEds, and Cox (28), has been shown by these and many other investigators to be a highly active carcinogen that attacks numerous sites in the rat and mouse. In addition, the parent hydrocarbon, fluorene, and several of its other derivatives have been tested for carcinogenic activity in the rat. Fluorene and its oxidation product, fluorenone, are inactive (30). While 2-nitrofluorene is only weakly carcinogenic (19) its reduction product, 2-aminofluorene, is highly active although somewhat less so than 2-acetylaminofluorene (2, 12, 19, 30). The activity of the latter compound is not greatly changed upon conversion to the diacetyl derivative (19). 7-Hydroxy-2-acetylaminofluorene, a metabolite of 2-acetylaminofluorene in the rat (3), has but little activity in this species (13)[1]. Similarly, the miscellaneous derivatives xanthone and 2-chlorofluorene are noncarcinogenic (30).

The objective of the study reported here was to determine if the $-CH_2-$ bridge in 2-acetylaminofluorene is essential to its carcinogenic activity in the rat. Three analogues were tested in which the bridges consisted of either -S- as in 3-acetylaminodibenzothiophene, -S- as in 3-acetylaminodibenzothiophene-5-oxide, or -O- as in 3-acetylaminodibenzofuran (see Table I for structures). The activity of 4-dimethylaminobiphenyl is also described. This compound, a derivative of 2-aminofluorene in which the bridge is absent, was originally tested as an analogue of the hepatic carcinogen 4-dimethylaminoazobenzene.

METHODS

Preparation of compounds.—2-Aminofluorene, prepared from fluorene[2] as described by Kuhn (14), was acetylated by dissolving it in excess hot acetic anhydride containing 1 to 2 per cent of redistilled pyridine. The 2-acetylaminofluorene was precipitated by the addition of 2 to 3 volumes of water, filtered, washed, and dried. It was then dissolved in hot ethanol, refluxed twice with 10 per cent of its weight of charcoal, and finally crystallized by the addition of water. The final yield, calculated from fluorene, was 50 per cent. The recrystallized product was nearly white and melted at 190°–191° C. 3-Acetylaminodibenzothiophene, m.p. 198°–199° C., and 3-acetylaminodibenzothiophene-5-oxide, m.p. 275°–276° C., were synthesized from dibenzothiophene as described previously (5). 3-Aminodibenzofuran, prepared by the method of Gilman and Avakian (9), was acetylated by heating for 1 hour on a water bath a benzene solution of the amine with a 10 per cent excess of acetic anhydride. The acetylated compound which precipitated from the solution was dissolved in hot alcohol and decolorized with charcoal. After recrystallization from 98 per cent ethanol the 3-acetylaminodibenzofuran melted at 179°–180° C. Since initial attempts to prepare 4-dimethylaminobiphenyl by methylating 4-aminobiphenyl with aqueous alkali and methyl sulfate (26) were unsuccessful, the methylation was carried out by adding 0.6 mole of dimethyl sulfate during a half hour period to 0.2 mole of 4-aminobiphenyl[3] while the temperature of the reaction mixture was maintained at 90° C. After allowing the mixture to cool to room temperature excess 10 per cent NaOH was added; the mixture was stirred for an hour and then filtered. The 4-dimethylaminobiphenyl melted at 120°–121° C. after being recrystallized twice from ethanol-acetone by the addition of water. The final yield was 30 per cent of theoretical; the product was recovered unchanged after refluxing with acetic anhydride.

Method of assay.—In the first series 6 male and 6 female albino rats,[4] 175 to 200 gm. in weight, were fed a

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[1] The control rats in this study were killed at 500 days while the rats fed 7-hydroxy-2-acetylaminofluorene were killed at 700 days.

[2] Reilly Tar and Chemical Corp., Indianapolis, Indiana.

[3] Eastman Kodak Co., Rochester, New York.

[4] Holtzman Rat Co., Madison, Wisconsin.

semi-synthetic diet (17, diet 3) with 1.80 millimoles of either 2-acetylaminofluorene or 3-acetylaminodibenzothiophene added per kgm. of diet (0.040 and 0.043 per cent, respectively) after solution in the corn oil with mild heat. For the second series (Table I) 10 male and 10 female rats, 165 to 190 gm. in weight, were fed diets containing 1.62 millimoles of either 2-acetylaminofluorene of 3-acetylaminodibenzothiophene (0.036 and 0.039 per cent, respectively) per kgm. of diet and 7 rats of each sex were fed 1.62 millimoles of either 3-acetylaminodibenzothiophene-5-oxide or 3-acetylaminodibenzofuran (0.042 and 0.037 per cent, respectively) per kgm. of diet. In this series a grain stock diet[5] was used, and the compounds were added as 10 gm. of a glucose mixture (prepared by grinding the compound and glucose together in a mortar) per kgm. of diet. After 8 months the feeding of the test compounds was discontinued, and the rats were maintained on the same grain diet. However, since no new tumors were observed in the 2 months following the removal of the carcinogen, the final incidences are recorded as of 8 months. In addition 10 female rats were also fed 1.62 millimoles of 3-acetylaminodibenzothiophene in the semi-synthetic diet used in the first series, but with the compound added in glucose. 4-Dimethylaminobiphenyl, after solution in the corn oil, was added to the semi-synthetic diet at a level of 5.33 millimoles per kgm. (0.105 per cent) and fed to 14 male rats for 8 months; these rats were then continued on the same diet without the carcinogen for an additional 2 months. This compound was tested in a series involving several azo dyes and was fed at a molar level twice that of 0.06 per cent 4-dimethylaminoazobenzene, the control carcinogen. In all series the rats of each sex were kept in separate screen-bottom cages in groups of 3 to 5. Food and water were available *ad libitum*.

The rats were autopsied at death or when death appeared imminent. Where the number of tumors available permitted, at least three gross tumors of each type from the rats fed each compound, all small tumors, and certain other tissues were studied histologically after being fixed in 4 per cent formalin, sectioned, and stained with hematoxylin and eosin. We are indebted to Dr. H. P. Rusch for the preliminary examination of the slides as reported here; a detailed description of these tumors will be presented later.

RESULTS

First series.—In this preliminary series 3 of the 6 female rats fed 3-acetylaminodibenzothiophene in the semi-synthetic diet developed tumors of the mammary glands after receiving the compound for 4 1/2 to 6 1/2 months. The other females and all of the males died tumor-free after receiving the compound less than 5 months. Tumors were found in the rats fed 2-acetylaminofluorene after the compound had been ingested for 8 to 11 months. Of the 6 females fed this diet one developed a tumor of the ear duct, another a liver tumor, and a third primary tumors of both the lungs and the ear duct. Of the 6 male rats fed this compound 3 developed tumors in the liver, and one of these rats also had a tumor of the ear duct.

Second series.—In this series the health and survival of the rats were better and the tumors developed earlier than in the first series. It is likely that this was due, at least in part, to the change from the semi-synthetic diet to the grain diet, since only 4 of the 10 female rats fed 3-acetylaminodibenzothiophene in the semi-synthetic diet survived for 4 months while 9 of the 10 females fed this compound in the grain diet were alive and in good health at this time.

The distribution of the tumors found in the rats fed the four compounds in the grain diet is given in Table I. Where more than one type of primary tumor was found on a single rat (for example, tumors from the ear duct and the mammary glands) each tumor is listed in the table. Although multiple tumors of one kind, particularly of the mammary glands and ear ducts, occured frequently they are not indicated. Where tumor incidences are presented in the text, they are based on the number of rats alive at 4 months. None of the tumors reported here has ever been observed when rats of this stock have been maintained for 12 months or longer on either the grain or semi-synthetic diets. After 15 to 20 months about 5 per cent of the female rats of this strain do develop spontaneous fibroadenomas of the mammary glands.

Each of these compounds induced tumors of the mammary glands in the female but not in the male rats. The first tumors were observed after 3 1/2 months and by 4 months the incidences were 67 and 57 per cent respectively for the rats fed 2-acetylaminofluorene and 3-acetylaminodibenzothiophene; the final incidences in these groups were 78 and 67 per cent respectively. In contrast, the sulfoxide of 3-acetylaminodibenzothiophene induced but one tumor at this site. While 3-acetylaminodibenzofuran finally gave rise to a high incidence of mammary tumors (83 per cent), the latent period was 2 to 3 months longer than for the other compounds. Other workers (2, 6, 12, 29) have also observed few or no tumors of the mammary glands in male rats fed 2-acetylaminofluorene, while these tumors usually develop in 30 (2, 6 12, 29) to 100 (8) per cent of the female rats. Mammary tumors from 2 rats fed 2-acetylaminofluorene and from 5 rats fed 3-acetylaminodibenzothiophene were aseptically removed, minced in saline, and injected subcutaneously into 4 to 6 young female rats. With the exception of one tumor from a rat fed the thiophene derivative, successful transplantation

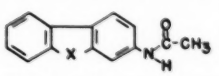
[5] Ground yellow corn, 68; linseed oil meal, 16; powdered skim milk, 12; alfalfa leaf meal, 2; cod liver oil, 1; NaCl (iodized), 0.5; and $Ca_3(PO_4)_2$, 0.5.

was obtained in 25 to 100 per cent of the new hosts. Second generation transplants were attempted and successful in each of two cases.

2-Acetylaminofluorene and 3-acetylaminodibenzothiophene were also equally effective in inducing tumors of the ear ducts while 3-acetylaminodibenzothiophene-5-oxide and 3-acetylaminodibenzofuran were less active in this respect. Thus, by 6 months 22 to 37 per cent of the male and female rats fed the first two compounds had developed this type of tumor; these incidences rose to 33 to 75 per cent by 8 months. Only 14 and 23 per cent of the rats fed 3-acetylaminodibenzothiophene-5-oxide and 3-acetylaminodibenzofuran respectively developed these tumors. The incidence of this type of tumor did not seem to be influenced appreciably by the sex of the animals. While this type of tumor has been consistently observed in other experiments with 2-acetylaminofluorene (2, 12, 29), the incidence in this experiment was greater than has been reported previously. The mammary and ear duct tumors induced by each of the three new compounds were very similar, grossly and histologically, to those induced by 2-acetylaminofluorene.

In contrast to the ability of each of the four compounds to induce tumors of the ear ducts and mammary glands only 2-acetylaminofluorene gave rise to liver tumors and gross liver damage. In line with observations by other workers (2, 12, 25, 29) the livers of male rats appeared to be more susceptible to this carcinogen than the livers of female

TABLE I

THE CARCINOGENIC ACTIVITIES OF ANALOGS OF 2-ACETYLAMINOFLUORENE WITH THE GENERAL FORMULA 

TUMOR SITE	MOS.	COMPOUND FED (1.62 MILLIMOLES/KGM. GRAIN DIET)							
		2-ACETYLAMINOFLUORENE X = -CH_2-		3-ACETYLAMINODIBENZOTHIOPHENE X = -S-		3-ACETYLAMINODIBENZOTHIOPHENE-5-OXIDE X = -SO-		3-ACETYLAMINODIBENZOFURAN X = -O-	
		F	M	F	M	F	M	F	M
		NUMBER OF RATS							
SURVIVAL*	4	9/10	10/10	9/10	8/10	7/7	7/7	6/7	7/7
MAMMARY GLAND	4	6	0	5	0	1	0	0	0
	6	6	0	6	0	1	0	2	0
	8	7	0	6	0	1	0	5	0
EAR DUCT	4	1	0	0	0	0	0	0	0
	6	2	3	3	3	1	0	0	1
	8	4	6	3	6	2	0	2	1
LIVER	6	0	1	0	0	0	0	0	0
	8	0	7	0	0	0	0	0	0
NONE†	4	3(3)	10(10)	3(3)‡	8(8)	6(6)	7(7)	6(6)	7(7)
	6	2(2)	7(7)	1(1)	5(5)	5(2)	7(7)	4(4)	6(6)
	8	0	0	1(0)	2(0)	4(0)	6(6)‡	1(1)	6(5)

*NUMBER LIVING AT 4 MONTHS OVER NUMBER AT START

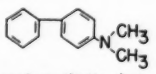
†TOTAL NUMBER OF TUMOR-FREE RATS (NUMBER OF TUMOR-FREE RATS STILL ALIVE)

‡ONE RAT, NOT INCLUDED HERE, DEVELOPED A TUMOR AT ANOTHER SITE (SEE TEXT)

rats. Although 6 female rats survived more than 6 months and 2 of these more than 8 months, none developed detectable liver tumors, while the incidence for the male rats was 70 per cent at 8 months. Further, the gross damage was usually less in female than in male rats fed 2-acetylaminofluorene for the same length of time.

In addition to tumors of the ear duct, mammary gland, and liver, single tumors were also found at three other sites. One female rat fed 3-acetylaminodibenzothiophene for 4 months developed an "oat-cell" carcinoma of the lung. This tumor was first discovered as a metastasis in the spleen, and one month after removal of the spleen the animal died with numerous metastases in the liver and mediastinum. A tumor of endothelial origin was found in the subcutaneous tissue over the forehead

TABLE II

The Carcinogenic Activity of 4-Dimethylaminobiphenyl, 

(fed at level of 0.105 per cent in semi-synthetic diet to 12 male rats for 8 months)

Tumor site	Diagnosis	Months 8 (no. of rats)	10 (no. of rats)
Mammary gland	adenocarcinoma	1	4
Mediastinum	sarcoma	0	1
Ear duct	epidermoid carcinoma	0	3
Liver	benign hepatoma	1	3
Vertebral canal	fibroma	0	2
None		11	3

of a male rat fed 3-acetylaminodibenzothiophene-5-oxide for 8 months. An epidermoid carcinoma of low grade malignancy arising from or near the vagina was found on autopsy of a female rat fed 3-acetylaminodibenzofuran for 8 months. Although this rat also had mammary gland and ear duct tumors, all of the three tumors were primary cancers.

4-Dimethylaminobiphenyl.—Twelve of the 14 male rats fed 4-dimethylaminobiphenyl survived for at least 8 months, the time at which the first tumor was found (Table II). By 10 months, 3 of the 12 rats had died without developing tumors. Between 8 and 10 months, 13 primary tumors were found in the remaining 9 rats. These included 4 adenocarcinomas of mammary gland origin, 1 sarcoma arising in the mediastinum and invading through the sternum, 3 tumors of the ear duct, 3 benign liver tumors, and 2 fibromas arising in or near the vertebral canal. The fibromas were found in 2 rats which developed a paralysis of one of the hind legs. Severe urine retention was present in these rats during the last week of life. These effects were presumably due to pressure by the tumor on the cord. The occurrence of mammary tumors in these male rats is of interest since none of the male rats fed the other compounds developed this type of tumor.

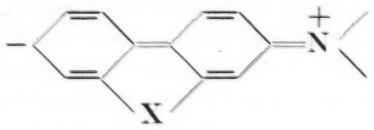
DISCUSSION

The data presented in Table I demonstrate that the substitution of a thio ether linkage, -S-, for the methylene linkage, $-CH_2-$, in the 2-acetylaminofluorene molecule did not alter its carcinogenicity toward the mammary gland and ear duct tissue of the rat. On the other hand, the introduction of the sulfoxide linkage, $-\overset{\overset{O}{\|}}{S}-$, produced a compound which was only feebly carcinogenic. Replacement with the ether linkage, -O-, led to a compound which eventually exhibited a high activity towards the mammary gland and a somewhat reduced activity towards the ear duct tissue. The inability of each of the three analogues of 2-acetylaminofluorene to induce tumors in the liver is noteworthy since the latter compound is quite active in this respect.

Somewhat similar observations have been made on the substitution of oxygen for sulfur, or *vice versa*, in several naturally occurring molecules. Thus the replacement of -S- by -O- in the ring of the biotin molecule does not seriously alter the ability of the resulting compound to act as a vitamin for several microorganisms as well as for certain higher species (1, 15, 31). However, oxidation of biotin to the sulfone destroys the ability of the molecule to act as a vitamin and, in fact, the sulfone can serve as a biotin antagonist (7). Conversely, the insertion of -S- for -O- in the ether linkage in thyroxine yields a molecule which can still accelerate metamorphosis in tadpoles (11). On the other hand, in methionine where the lability of the $S-CH_3$ bond is important to its activity *in vivo*, the replacement of -S- by -O- leads to an antimetabolite (24).

The carcinogenic activity of 4-dimethylaminobiphenyl is of considerable interest; for even though the activity of this compound, on both a time and molarity basis, was relatively low, it induced a variety of tumors in a high percentage of the surviving animals. Of particular interest is the fact that this compound induced mammary tumors in male rats; such tumors are rare in male rats fed 2-acetylaminfluorene (2, 6, 12, 29). The sites which this molecule attacks show that it is more closely related to the aminofluorenes than to the aminoazo dyes. In comparison with the former

class the -CH_2- bridge is missing and in comparison with the latter class the azo, -N=N-, linkage is absent. However, the omission of the bridge in the fluorene molecule is probably the less serious of the two since here the inter-atomic distances are probably not greatly changed. This follows from the fact that biphenyl and some of its derivatives generally tend to assume a coplanar configuration (27) which is, very probably, rigidly maintained in the fluorene molecule. Arguments have been presented in favor of this uniplanar structure (23) as well as a labile folded ring structure for fluorene (22). The present authors consider it possible that the greater biological activity of the fluorene derivatives *versus* the biphenyl derivative (Tables I and II) is associated with the -CH_2-, -S-, and -O- groups which help maintain a coplanar arrangement of the benzene nuclei. One consequence of this arrangement is that the structure of the molecule would then be represented to a greater extent by resonating quinonoid structures such as:



It is also interesting that Haddow *et al.* (10) found 4-dimethylaminobiphenyl to be inactive as a tumor inhibitor. Apparently it is an exception to the approximate correlation which Haddow and his group have observed to hold between the growth-inhibitory power and the carcinogenicity of certain polycyclic aromatic hydrocarbons. Later these studies led to the discovery of the carcinogenic activity of 4-dimethylaminostilbene and some of its derivatives. In this connection it is notable that while the 4-aminostilbenes are isosteric with the 4-aminoazo dyes, they have carcinogenic activities (10) which ally them more closely with 2-acetylaminofluorene and 2-anthramine (4). All of these compounds, including 4-dimethylaminobiphenyl, induce tumors in a variety of sites and have in common the ability to produce tumors of the ear duct tissue. The aminoazo dyes, however, induce only liver tumors when fed to rats.

The data presented in this paper bear on the hypothesis put forth by Pinck (20, 21) in his attempt to explain chemical carcinogenesis. According to Pinck the -CH_2- bridge of 2-acetylaminofluorene is of prime importance in a series of dehydrogenation and condensation reactions between this carcinogen and a hypothetical tissue component $H_2C\begin{matrix}X\\Y\end{matrix}$. Through a catalytic process this reaction is thought to lead to the formation of a polymer, $\begin{matrix}X\\Y\end{matrix}CH-\left(C\begin{matrix}X\\Y\end{matrix}\right)_n-HC\begin{matrix}X\\Y\end{matrix}$, which then accumulates in the cell autocatalytically. Pinck suggested that the formation of such molecules constitutes the carcinogenic process and further postulated that "the difference between cancer tissue and normal tissue is that the former consists of a very long carbon chain of cell substance." In addition to the fact that the latter postulate suffers from a complete lack of supporting data it is difficult to understand how the -S- and -O- bridges in 3-acetylaminodibenzothiophene and 3-acetylaminodibenzofuran, respectively, could participate in the proposed reactions. The carcinogenic activity of 4-dimethylaminobiphenyl which has no bridge connecting the 2 and 2′ positions is even more difficult to explain on this basis. Aside from these objections we are in accord with the general idea that chemical carcinogens may act through combination with certain cell constituents, especially protein, and have presented evidence for this in the case of azo dye induced liver tumors (16, 18).

SUMMARY

1. The carcinogenic activities of 3-acetylaminodibenzothiophene, 3-acetylaminodibenzothiophene-5-oxide, and 3-acetylaminodibenzofuran were directly compared with that of 2-acetylaminofluorene in male and female rats. A fourth compound, 4-dimethylaminobiphenyl, was tested for carcinogenic activity in male rats. All of the compounds were fed for 8 months.

2. The data demonstrate that replacing the -CH_2- bridge in 2-acetylaminofluorene by -S- as in 3-acetylaminodibenzothiophene did not alter the carcinogenicity of the molecule for either mammary gland or ear duct tissue. Substitution of -S(=O)- for the -CH_2- as in 3-acetylaminodibenzothiophene-5-oxide greatly lowered the activity towards these two tissues, while insertion of an -O- bridge as in 3-acetylaminodibenzofuran only partially diminished the activity of the molecule in these respects. Unlike the control compound, 2-acetylaminofluorene, however, none of these 3 compounds had any carcinogenic activity towards the liver. The tumors produced by all of these compounds appeared 4 to 8 months after the beginning of the experiment.

3. 4-Dimethylaminobiphenyl, a derivative in which the -CH_2- bridge is absent, produced tumors in the mammary glands, ear duct, liver, and vertebral canal of male rats. These tumors appeared

8 to 10 months from the beginning of the experiment.

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Announcements

BRITISH AMERICAN EXCHANGE FELLOWSHIPS

British American Exchange Fellowships in Cancer Research of the American Cancer Society, awarded by the Society upon recommendation of the Committee on Growth of the National Research Council, are offered to citizens of the United States for advanced training and experience in Great Britain in specialized fields of investigation pertaining to the problem of cancer. These fellowships are awarded to provide specialized training for American investigators in Great Britain where opportunities exist for study in facets of research in malignant disease not widely available in this country. Training for an equal number of young British scientists selected by the Campaign will be provided in this country. Fellowships are open to citizens of the United States who possess the degree of Doctor of Medicine, Doctor of Philosophy, or Doctor of Science. They are intended for young men and women embarking on a career in clinical medicine or basic research, and also for more mature candidates desiring to extend their fields of competence in these fields. Application forms may be procured from and submitted at any time to the Executive Secretary of the Committee on Growth, Division of Medical Sciences, National Research Council, 2101 Constitution Avenue, Washington 25, D.C.

FUNDS FOR RESEARCH ON LYMPHATIC LEUKEMIA

The National Research Council announces the availability of a fund of $25,000 from the estate of Charles R. Blakely for support of research in the field of lymphatic leukemia. Applications for grants-in-aid from this fund are now being entertained. Application forms and additional information may be obtained from the Chairman, Division of Medical Sciences, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

CHANGE IN EDITOR: Dr. Harold P. Rusch will become Editor-in-Chief of *CANCER RESEARCH* beginning with Volume 10, January, 1950. All manuscripts submitted for publication should be sent to him, care of McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin, effective September 10, 1949.

AVAILABILITY OF GRANTS AND FELLOWSHIPS: The Committee on Growth of the National Research Council, acting for the American Cancer Society, is entertaining applications for grants and fellowships. Applications for new Grants in Cancer Research will be received until 1 October. Investigators now receiving grants will be notified individually regarding application for the extension of these grants. Final decision on applications will be made in most cases soon after 1 February. Grants approved at this time ordinarily will become effective 1 July 1950.

Fellowship applications may be submitted at any time. Those received prior to 1 November will be acted upon by the Committee on Growth in December. Those received between 1 November and 1 March will be acted upon in April. Fellowships ordinarily will begin 1 July though this date may be varied at the request of the applicant.

During the past year the American Cancer Society, Inc., on recommendation of the Committee on Growth has approved research grants and fellowships totalling over $2,000,000.

Communications regarding grants and fellowships should be addressed to Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.